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Species-specific drivers of West Nile virus transmission in urban environments

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

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Species-specific drivers of West Nile virus transmission in urban environments

By

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B.S., University of Georgia, 2009

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Abstract

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Heterogeneity, specifically biological variation, is an inherent component of pathogen transmission systems. Transmission variability arises due to differences in an individual's ability to acquire and transmit a pathogen (i.e. pathogen competence) and the probability infected individuals encounters susceptible individuals. For vector-borne pathogens, competence varies among host and vector species, and the composition of host and vector communities strongly influences the rate at which pathogens are transmitted among individuals. Because species community composition varies across the spatiotemporal landscape, species-specific drivers of transmission can vary from region to region.

The objective of my dissertation is to quantify vector and host species-level transmission heterogeneities and then link these dynamics across species using West Nile virus (WNv) as a model system**.** West Nile virus is a mosquito-borne, zoonotic pathogen that is transmitted by mosquito vectors in the *Culex* genus among birds. I specifically use a combination of experimental, field surveillance, and modeling approaches to quantify connections in WNv transmission by two *Culex* spp.*, Culex restuans* and *Culex quinquefasciatus*, and the diverse bird species communities upon which these two vectors feed. In Chapter 1, I use field surveillance of WNv in Atlanta, GA coupled with modeling techniques to show that climate and the availability of susceptible hosts mediate the likelihood that *Cx. restuans* and *Cx. quinquefasciatus* are efficient vectors of WNv. In Chapter 2, I use long term sero-surveys of wild birds in Chicago, IL and Atlanta, GA to quantify the variability of WNv incidence across sampled species. In Chapter 3, I extend results from Chapter 2 and use blood feeding experiments to show that the feeding behaviors of *Cx. quinquefasciatus* are a function of the availability of certain host species. Finally, in Chapter 4 I use an applied approach to show that targeted larvicide applications in road-side catch basins are insufficient to control WNv at local scales. All though each chapter addresses an important component of heterogeneity in the WNv transmission system, further research is needed to determine the extent to which the intensity of WNv transmission in the enzootic cycle translates to the risk of human WNv-incidence.

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Introduction

In ecology, heterogeneity is defined as biologically significant variation, and variation in any ecological system can arise from its functional and structural components (1). Functional heterogeneity is defined as variation within a biological process or property, while structural heterogeneity describes the composition of a community through space and/or time (often without direct reference to the process or property) (2). Importantly, functional and structural sources of heterogeneity are not mutually exclusive. For instance, variation in the structural composition of communities can influence functional processes such as population growth rates, competitive interactions, and parasitism (3). This nested variability limits generalizations regarding ecological processes to systems outside of the structural context in which they were defined (4). Thus, species interactions such as parasitism are highly localized events and heterogeneous across the landscape.

In this dissertation, I focus on identifying and measuring sources of heterogeneity attributable to particular species in the West Nile virus transmission system in the southeastern United States (U.S.). West Nile virus is a mosquito-borne virus within the family *Flaviviridae* that also contains the Japanese encephalitis virus and St. Louis encephalitis virus (SLEv) groups (5, 6). These viruses are transmitted among non-human vertebrates by mosquitoes in the *Culex* genus; WNv is predominately transmitted by members of the *Cx. pipiens* complex among birds (5, 7). Importantly, humans and mammals are considered dead-end hosts of the virus because mammals are incapable of producing viremia in their blood sufficient to infect a susceptible mosquito (5). However, WNv infections can be pathologically severe in elderly and immune-suppressed humans as well as in horses (8).

West Nile virus was first described in 1937 in Uganda, and there are reports of human outbreaks in Eastern Europe and Africa in the following decades (5, 9). The virus was then introduced to North America in 1999 (first detected in New York City, NY, U.S.) from whence it spread throughout the U.S. reaching California and the Pacific coast by 2004 (5). During the invasion phase of WNv, native North American bird species were fully susceptible to WNv which led to massive die offs of bird populations throughout the country (10, 11). Spillover of the virus into human populations was also widely prevalent during the invasion phase (8). West Nile virus is now considered an endemic wildlife arbovirus in the U.S., and annual reports of WNv infections in humans vary across the country (6, 8).

The primary vectors of WNv in North America are members of the *Cx. pipiens* complex. This species complex includes a diverse mix of subspecies and hybrids, including: *Culex pipiens pipiens* Linnaeus (distributed in Northern latitudes), *Culex quinquefasciatus* Say (distributed in southern latitudes), *Culex pipiens molestus* Forskål that breeds primarily underground, and hybrids of the subspecies where distributions overlap (12-16). The complex is classified as the primary vectors of WNv because its members broadly feed on birds (with the exception of the *molestus* form which feeds more commonly on mammals), are commonly found infected in the field during WNv epidemic periods (5), and experimental studies have demonstrated the dynamics of infection in its members (17-20). West Nile virus infections have also been reported in many other mosquito species (21), including but not limited to *Culex tarsalis* Colquiett in the western U.S. (22, 23), *Culex restuans* Theobald in the eastern U.S. (19, 24, 25), and *Aedes albopictus* Skuse (where its distribution overlaps with more efficient vector species). *Culex tarsalis* is considered an epidemiologically important primary vector within its distribution (17, 22), while the other species are considered secondary vectors. This is either because the

spatial/temporal distributions of secondary vector species reduce encounters with infectious hosts and/or their blood feeding behaviors reduce their likelihood of obtaining a blood meal from an infected bird species. Importantly, the general absence of detected WNv infections in secondary vector species from the field limits their classification as epidemiologically important vectors (26).

Although birds are the primary hosts of WNv, infection dynamics vary greatly between species. Early WNv experimental infection studies in the U.S. identified predominately Passerine species as the most competent host species of the virus: blue jays (*Cyanocitta cristata*), common grackles (*Quiscalus quiscula*), American crows (*Corvus brachyrhynchos*), house sparrows (*Passer domesticus*), and American robins (*Turdus migratorius*) were all identified as competent hosts of WNv (27). During the invasion phase of WNv in North American, blue jays and American crows were commonly reported deceased by members of the public participating in local dead bird surveillance programs (11, 28, 29). Other passerine species such as northern cardinals (*Cardinalis cardinalis*) and northern mockingbirds (*Mimus polyglottos*) are susceptible to WNv infection yet are only mildly infectious to mosquitoes (30). High WNv antibody titers (an indicator of either severe infections or robust immune responses) have been reported in certain Raptor species (31, 32). In general, however, birds of prey, game birds (such as quail and pheasants), Anatidae (ducks and geese), and woodpeckers are considered incompetent or uncommon hosts of WNv (27). Additionally, observational studies show a negative correlation between WNv infection prevalence in mosquitoes and habitats dominated by non-Passerine species (33).

Spatially, WNv is primarily an urban mosquito-borne pathogen, although transmission cycles occur were appropriate vector and host species overlap (34). The intensity of transmission

in urban centers may be due to the spatial aggregation of competent host and vector species (35). West Nile virus infection rates in mosquitoes in urban environments are also positively associated with landscape features such waste water treatment facilities (29) and housing age (36). Human behaviors are also an important, but commonly under-investigated, risk factor of encountering WNv infected mosquitoes as well as other similar arboviruses; previous studies of SLEv linked lower rates of human incidence to increasing household ownership of televisions and air conditioning units (37). Epidemic spillover of WNv in humans appears to be limited to temperate regions of the globe, with a noticeable lack of human cases reported in tropical climates (38). Cross-reactivity of antibodies between WNv and other tropically circulating viruses may be one reason for this lack of human incidence (39); underreporting of human cases and variation in host selection behaviors of *Cx. pipiens* complex mosquitoes may also explain this observed pattern.

Temporally, WNv infections are most prevalent in host and vector communities in the summer months as is incidence of spillover in humans: in North America those months are June through September/October. West Nile virus Infection prevalence in mosquitoes is positively associated with high summer temperatures as well as drought severity (40-43). High summer temperatures accelerate the extrinsic incubation rate of WNv within infected vectors (44, 45) while drought severity leads to aggregations of hosts and vectors near water sources (46, 47). Aggregated infection prevalence in the summer months may also be driven by the seasonal abundance of both hatch year birds (48) and local populations of primary *Culex* spp. mosquitoes. How WNv populations survive from one epidemic period to the next is still under investigation, though researchers have identified chronic infections in hosts (49, 50) and viral overwintering in

diapausing *Cx. pipiens pipens* mosquitoes (51-53) as possible mechanisms of inter-epidemic pathogen survival.

Ecologically, when species vary in their intrinsic susceptibility and infectiousness to a pathogen, the structural composition of host and vector communities can influence pathogen transmission rates and the prevalence of infections among vector and host species (54, 55). In North America, the structural composition of local host and vector species communities varies substantially from region to region such that species-specific contributions to WNv transmission vary across the U.S. In order to identify species-specific contributions to transmission, WNv must be investigated at local scales.

West Nile virus in the southeastern U.S.

Previous work on WNv transmission in the southeastern U.S. by my collaborators has investigated the variability of WNv transmission in urban microhabitats in relation to understanding risk factors associated with human and mammalian spillover (29, 56-62). A primary finding from this previous body of research is the influence of host reservoir competence on rates of enzootic WNv transmission.

Using field surveillance of WNv from 2010 – 2011 in Atlanta, GA, Levine et al. (2016) found that northern cardinals have the highest WNv antibody prevalence among sampled bird species (57); northern cardinals were also the most commonly sampled species with detectable levels of WNv viremia in their blood (60). Additionally, northern cardinals were the most common blood meal host of *Culex* spp. mosquitoes in a survey of mosquito blood feeding in Atlanta. However, due to northern cardinals low reservoir competency for WNv, northern cardinals provided a low amplification fraction of WNv; a similar finding was reported for

species in the Mimidae family (including brown thrashers, northern mockingbirds, and gray catbirds) (57).

Among bird species identified as a blood meal host for *Culex* spp. mosquitoes, American robins had the highest amplification fraction for WNv due to this species high reservoir competence for WNv (57). Levine et al. (2016) additionally observed a temporal shift in *Culex* spp. blood feeding on American robins to northern cardinals during the epidemic phase of WNv (July to September) (57), and proposed that: 1) enzootic WNv transmission in the Atlanta region is driven by northern cardinals, and 2) *Culex* spp. feeding shifts to northern cardinals and members of the Mimidae family during WNv epidemic periods depress the amplification of WNv in the region to levels insufficient to result in human spillover of the virus (56, 57, 60).

This work confirms reports from a previous seroprevalence survey in the State of Georgia that indicated that northern cardinals had the highest WNv antibody prevalence among sampled species (63). Levine et al.'s research was also the first eco-epidemiological study in the State of Georgia to comprehensively investigated the influence of microhabitats on WNv infection prevalence in mosquitoes and seroprevalence rates in birds. A primary result of this aspect of her research was that heavily forested habitats in the city are negatively associated with WNv antibody prevalence in birds and WNv infection rates in mosquitoes (57, 60).

Dissertation objectives

Though the methods employed in my dissertation are founded in these previous field studies of WNv in Atlanta, GA, the intent of my research is to link empirical data on WNv infection prevalence in vector and host communities to hypotheses regarding both species-specific contributions to WNv enzootic transmission and general ecological theories of vector-

borne pathogen transmission. The objective of my dissertation is to directly quantify WNv transmission heterogeneity in the southeastern U.S. caused by variability in vector species community composition (Chapter 1), host species community composition (Chapter 2), and the blood feeding behaviors of *Cx. quinquefasciatus* (Chapter 3); I also present research related to an applied field test of vector control methods (Chapter 4). Specifically, I use a combination of field surveillance, experimental, and theoretical methods to investigate sources of heterogeneity in the WNv system. Though each chapter addresses an important component of variability in the WNv transmission system, further research is needed to determine to what extend the intensity of WNv transmission in the enzootic cycle translates to the risk of human WNv-incidence.

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Chapter 1: Linking transmission potential of multiple vectors to observed patterns of pathogen transmission

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Introduction

Identifying sources of pathogen transmission heterogeneity is difficult because not all individuals or groups within a population contribute equally to transmission (1). For vector-borne pathogens in field settings, it is common practice to ignore individual level contributions to transmission and instead researchers focus on identifying a primary vector and host species most responsible for transmission (2). Primary vector species are classified by their ability to acquire and transmit infections among hosts and their propensity to feed on pathogen competent hosts. Epidemiologically, the vectorial capacity model incorporates these attributes in a single equation that estimates a vector species' potential to spread the pathogen (3). Specifically, vectorial capacity uses four main parameters to predict the likelihood a vector species is an efficient vector of a pathogen: vector density, vector-host contact rates, vector mortality rates, and the extrinsic incubation period of the pathogen (4). Vectorial capacity provides a theoretical estimate of the number of infectious vectors generated from a population feeding on a single infected host per unit area and time (3). Each parameter provides a point of attack for vector-borne pathogen control, and the use of vectorical capacity as an epidemiological tool has led to the successful management of mosquito-borne human pathogens such as malaria and dengue (5).

Despite the eco-epidemiological focus on controlling pathogen transmission by targeting interventions at primary vector species, there is increasing theoretical evidence that non-primary, or secondary, vector species may directly or indirectly contribute to transmission. An underlying assumption of the vectorial capacity model is that transmission is independent between species, and therefore total vectorial capacity of a community of vectors is the sum of each vector species' estimate (4). Thus, the null prediction of the vectorical capacity model is that increasing the number of transmitting vector species will lead to a linear increase in total transmission potential. In order for this to be the case, added vector species must be competent for the pathogen as well as overlap in host usage (6). Where different competent vector species occur at different times of the year, transmission may be interdependent among vector species such that non-linear increases in transmission occur due to: 1) the increased presence of competent species through time may extend the length of a transmission season (7) and/or, 2) the increased presence of competent species may reduce the probability of pathogen extinction during inter-epidemic periods (8).

Extended transmission seasons and reduced probability of pathogen extinction are relevant to numerous vector-borne pathogen systems. For example, *Anopheles coluzzi* Coetzee & Wilkerson sp. n. and *Anopheles gambiae* Giles in the African Sahel differ in their dry season survival such that *An. coluzzi* is the most abundant malaria vector at the beginning of the wet season and *An. gambiae* becomes the more abundant vector as the wet season progresses (9). For the avian arboviruses St. Louis Encephalitis virus and West Nile virus (WNv) in temperate North America, early season *Culex restuans* Theobald populations are thought to act as amplifying vectors that restart the arbovirus' transmission season after an overwintering period; populations of *Culex pipiens* complex mosquitoes then become the primary epidemic vectors as

their population abundance grows and the transmission season progresses (10, 11). Multi-vector species transmission in these systems may be independent or interdependent, and it is the objective of this report to investigate the dynamics of multi-vector species transmission in the WNv system.

West Nile virus is a zoonotic, mosquito-borne virus transmitted by multiple *Culex* spp. mosquitoes among birds (12). In North America, the primary vectors are members of the invasive *Cx. pipiens* complex including *Culex pipiens pipiens* Linnaeus in northern latitudes and *Culex quinquefasciatus* Say in southern latitudes; in the Western U.S. native *Culex tarsalis* Coquillett mosquitoes are also considered primary vectors (13). Native *Cx. restuans* populations in the eastern U.S. preferentially feed on birds and are competent vectors of WNv in laboratory settings (14). However, because *Cx. restuans* populations reach peak abundance in the field during non-epidemic periods (i.e., spring), the species is considered a secondary vector of WNv (12). We hypothesized that early season *Cx. restuans* populations may be important early season amplifying vectors of WNv, and that the species' early season abundance extends the length of the WNv transmission season (10, 11), possibly resulting in a non-linear contribution to transmission.

To test our hypothesis, we developed a simple temperature-dependent vectorial capacity model for WNv and then linked this model to empirical field evidence of WNv transmission in the southeastern United States. We provide time-varying estimates of transmission potential that can be helpful for disentangling the relative contributions to pathogen transmission of primary and secondary vector species with overlapping life history traits.

Materials and Methods

The WNv vectorial capacity model

We define vectorial capacity (C) as the number of infected mosquitoes expected from mosquito populations feeding on a single infected host. The model's formulation is

$$C = - m*a^2*P^{EIP} / \log(P)$$

where m = vector density, a = 1/gonotrophic period (i.e., the daily rate of successfully blood feeding), P = daily survival probability, and EIP = the extrinsic incubation period (5). The life history traits of *Cx. restuans* and *Cx. quinquefasciatus* relevant to WNv transmission are similar; both species feed on birds (24), are competent lab vectors of WNv (14), and are often collected in the same habitats (11). Because of these similarities and because at this time there are no published biologically significant differences between each species' biting rates and EIP, we assumed that *Cx. restuans* and *Cx. quinquefasciatus'* WNv transmission potential was driven by the differences in each species' field abundance. Our vectorial capacity model builds on the current hypothesis in the literature that *Cx. restuans'* contribution to WNv transmission occurs primarily through its early season field abundance (10, 11).

Daily survival probabilities, *P*, were estimated by fitting a linear model to mortality rates of *Cx. pipiens pipiens*, *Cx. quinquefasciatus*, and *Cx. restuans* field populations at different temperatures published in (25). The model of mortality as a linear function of temperature is: (1/median survival time) (i.e. mortality rate) = 0.0056**Temperature*. Ciota et al. reports variation in *Culex* spp. survival at different temperatures; however, we chose to use the linear fit through all published survival estimates for the investigated field populations (**S. Fig. 1**). Then, the daily probability of vector survival was generated as $\exp(^{-\text{Mortality Rate}})$.

Biting rates, *a*, were estimated via a published equation for the gonotrophic period for *Culex* spp. mosquitoes (17). The model is: $a = 1 / (n + (1/-0.066 + 0.018*Temperature))$. The equation asymptotes at 3.7°C which is considered a thermal minimum for successful blood meal digestion in *Cx. tarsalis*. In the denominator, *n* units in days are added to the equation to account for the time it takes for a vector to locate a host and an oviposition site. Because birds and breeding sites are widely available to *Culex* spp. mosquitoes in our sample sites, we assumed these questing intervals totaled 1 day. Prior usage of this equation assumed these questing intervals to be 2 days (26). Importantly, this equation assumes only one successful blood meal per gonotrophic period. Units are the number of successful bites per day.

The extrinsic incubation period, *EIP*, was modeled using the published equation in (27) for WNv incubation in *Cx. tarsalis*. The model is: $EIP = 1/ (-0.132 + 0.0092*Temperature)$. This equation estimates the number of days it takes for a WNv-exposed mosquito to become infectious. The equation asymptotes at 14.3° C, which is assumed to be a thermal minimum for WNv replication within *Cx. tarsalis*; similar temperature-dependent incubation periods have been reported for *Cx. pipiens* complex mosquitoes (28). Units are in days.

Vectorial capacity estimates greater than 1 indicate that pathogen spread is likely and represents a theoretical threshold value for transmission potential. Under our construction of vectorial capacity, we investigated the minimum temperature and vector densities at which capacity estimates exceeded 1. To define these minimum thresholds, we generated capacity estimates across a temperature range of 14.5 to 40° C and vector/host densities ranging from 1 to 100. We then assessed how changes in a single parameter altered the minimum temperatures and vector densities at which capacity estimates exceeded 1. These theoretical capacity estimates

were used to quantify under what combinations of parameters *Cx. restuans* and *Cx. quinquefasciatus* would be likely vectors of WNv.

WNv mosquito surveillance

From April 2014 through December 2016 we sampled mosquito and bird communities for evidence of WNv transmission in four sites in Atlanta, GA with historical evidence of WNv enzootic transmission (15, 16). Grant Park (GP), in central Atlanta (**S. Fig. 2**), was our primary surveillance site for all three years. Phoenix Park (P3; sampling began June 2015) and Springvale Park/Inman Park (SVP and IMP, respectively; sampling began March 2016) are public spaces near GP with similar ecological attributes (**S. Fig. 2**). Additionally, road-side catch basins, the dominant breeding sites for *Culex* spp. mosquitoes in urban environments (17), were widely distributed within and along the boundaries of all four parks.

Mosquito WNv surveillance included weekly collections of adult mosquitoes within catch basins coupled with collections of gravid female mosquitoes using CDC gravid traps (18). Adult mosquitoes were sampled from the interior of catch basins for up to 5 minutes with a handheld Prokopack aspirator (19), with the same 8 to 10 catch basins sampled at each site during each sampling period. Additionally, 3 to 4 gravid traps were set overnight within 200 m from catch basins at each collection site. Catch basin and gravid trap collections were returned to Emory University for morphological identification using a dichotomous key (20). All *Culex* spp. and non-*Culex* spp. female mosquitoes were pooled for virus testing by date, collection method, site, and species with up to 25 individuals per vial. Pools were tested for WNv using previously described virus isolation techniques (16). Briefly, pools were homogenized with a tissuleyzer then centrifuged at 10,000 rpm for 10 minutes. Then, 100µl of the supernatant was plated on to

Green Monkey kidney cells in cell culture media. Cells were incubated at 37°C for 3-5 days and monitored for cytopathic effects (CPE). West Nile virus infections in cells showing evidence of CPE were confirmed using RT-PCR and Vec-Tests (Fisher Scientific ©). Minimum Infection Rates (MIR) per 1,000 individuals were calculated using the PooledInfRate Excel plug-in (21).

WNv avian surveillance

Bird sampling protocols were approved under USGS Permit 23673, GA DNR Scientific Collection Permit #23772, and Emory IACUC approval DAR-2003079.

Bird populations were sampled weekly from each site to monitor WNv antibody prevalence, an indicator of prior WNv exposure. Birds were collected using mist nets following a protocol developed by (16). Mist netting took place from approximately 0600 to 1300 hrs in the absence of precipitation and high wind speeds (> 15 km/h). Captured individuals were identified to age, sex and species following (22). Up to 200 µl of blood was collected via jugular venipuncture from birds weighing > 15 g and in suitable physical condition (e.g., no injuries or signs of severe stress). Blood samples were returned to Emory University, centrifuged for 10 min at 10,000 rpm, and serum was separated from blood clots.

All serum samples were tested for IgY (an avian immunoglobulin functionally similar to the mammalian IgG) antibodies to WNV using a virus neutralization procedure following Chapter 2.1.24 of the OIE Diagnostic manual for terrestrial animals (23). Briefly, sera were heat inactivated at 30 minutes at 56°C and then serially diluted, starting at 1:4 - 1:4096 in cell-culture media. First equal parts virus (100-300 $TCID_{50}$ per 1 ml) and serum (50µl each) was added to the culture and incubated at 37°C. After one-hour incubation, 100ul of cells were added and plates were incubated at 37°C for 3-5 days and read for cytopathic effects (CPE). Endpoint titres were

based on the last well to display complete protection against CPE. Negative and positive controls were run as well as a cell control and virus back titration control to ensure testing integrity. Titers equal to or greater than 1:8 were considered antibody positive. Sera were also screened for WNv viremia by depositing 8 µl of serum onto cell-culture plates with Green Monkey kidney cells and then following procedures described above for mosquito infection.

Linking vectorial capacity to evidence of WNv transmission

We generated field estimates of vectorial capacity for *Culex* spp. mosquitoes collected in Atlanta, GA, by first estimating biting rates, survival, and EIP using the average weekly temperature observed during each week of mosquito sampling. Because static estimates of capacity assume vector populations are comprised of recently emerged, susceptible females (4), we chose to limit our density estimates, *m*, to adult female collections in catch basins rather than gravid traps. Because we did not estimate avian population abundances in relation to vector abundance, all reported catch basin collections are assumed to be the number of vectors emerging from a basin per host. Temperature data are from the National Oceanic and Atmospheric Administration field station at Hartsfield-Jackson International Airport. Capacity estimates were generated per catch basin per collection week.

Data Analysis

Mixed effects models were used due potential positive correlations between repeated spatial and temporal measurements. Additionally, because not all sites were sampled equally across all three years, we analyzed two subsets of the entire data set: GP only and 2016 collections only. The type of outcome defined the format of each model. We used Poisson-errors generalized linear mixed models (GLMM) to compare *Culex* spp. collections in catch basins

between months (both data sets), sites (2016 data set), and years (GP data set) using catch basin as a random effect. To compare capacity estimates, we first transformed our capacity estimates by adding 0.01 and taking the log, yielding values more closely following a Gaussian distribution. We then implemented Gaussian-error LMMs on the transformed outcomes with catch basin random effects. Average daily air temperature was included as a covariate in all mosquito models. Month was centered to July and temperature was centered to the average value in the data set (22.93° C) to improve model convergence for all models. We next used binomial-error GLMMs to compare avian WNv antibody prevalence by bird age, month of collection, year (GP data set), and Park (2016 data set) with avian species as a random effect. For within GP analyses, 2014 was the reference year and in 2016 analyses GP was the reference site. GLMMs were run using the 'glmer' functions in the R package 'lme4' and LMMs were run using the 'nlme' package in R. All analyses were performed in R V 3.4 (29).

Results

Vectorial capacity thresholds

The minimum vector density at which capacity estimates exceeded 1 was four vectors per host when temperatures exceeded 33.4° C (**Fig. 1A**). As vector densities increased the thermal minimum of the capacity model decreased at an exponential rate. Changes at low vector density had the greatest proportional effect on the thermal minimum. For example, an increase in vector density from 5 to 10 lowered the thermal minimum from 28.67° C to 21.96° C while a further increase in vector density to 15 only reduced the thermal minimum to 20.17° C (**Fig. 1A**). Importantly, very high feeding rates (> 0.7 feeds per day) (**Fig. 1B**) and survival probabilities (>

0.91) (**Fig. 1C**) at appropriate temperatures could reduce the minimum vector density at which capacity estimates exceeded 1 to a single blood-feeding vector.

Field estimates of vectorial capacity

Observed daily average temperatures were highest in June through August (**S. Fig. 3A**) which translated to high predicted biting rates (**S. Fig. 3B**), low daily survival probabilities (**S. Fig. 3C**), and short extrinsic incubation periods (**S. Fig. 3D**). Increases in air temperature were also associated with increased *Culex* spp. collections within basins and increased capacity estimates in all models (**Table 1 and 2**), most likely reflecting temperature's modeled relationship with the parameters of the vectorial capacity model.

Over 90% of all *Cx. restuans* individuals were collected in GP, and the number of months into the collection season had a negative effect on *Cx. restuans* collections, reflecting the fact that this species was most abundant early in each transmission season (**Table 1, S. Fig. 4**). Within the GP data set, there were no differences in *Cx. restuans* collections in catch basins between years (**Table 1**). There were fewer total *Culex* spp. mosquitoes (i.e. the sum of all identified and unidentified *Culex* spp. individuals) collected in catch basins during 2015 and 2016 compared to 2014 **(Table 1**). Additionally, month had a negative effect on total *Culex* spp. catch basin collections within GP, most likely reflecting that the population abundance for *Culex* spp. communities peaked between June and August and declined from August into December (**S. Fig. 4A and 4B**). Within the 2016 data set, fewer *Cx. restuans* mosquitoes were collected in IMP, P3, and SVP compared to GP (**Table 1**). There were no significant differences in *Cx. quinquefasciatus* collections within catch basins between GP and IMP (**Table 1**). Overall fewer

Culex spp. mosquitoes were collected in catch basins in IMP, P3, and SVP compared to GP (**Table 1**).

Differences in *Culex* spp. capacity estimates reflected differences in each species abundance, and thus total capacity estimates from GP were lower in 2015 and 2016 than in 2014 (**Fig. 2A, Table 2**); in 2016 total *Culex* spp. capacity estimates were lower in IMP, P3, and SVP compared to GP (**Fig. 2B, Table 2**). Within GP, peak *Cx. restuans* capacity estimates occurred in May and June (mean: 0.34, range: 0.22 – 0.6) (**Fig. 2E**). Because *Cx. restuans* populations were low to absent in IMP, SVP, and P3, mean *Cx. restuans* capacity estimates in these sites in the month of May was 0.03 (range 0.008 – 0.05) (**Fig. 2F**). Only in May 2015 were *Cx. restuans* capacity estimates greater than *Cx. quinquefasciatus* estimates in GP (Wilcoxon Test, $W = 514.5$, $p < 0.01$) (**Fig. 2E**). Capacity estimates for *Cx. quinquefasciatus* were equal to or greater than *Cx. restuans* capacity estimates at all other months in the GP and 2016 data sets (Wilcoxon Test, $W = 509670$, p-value < 0.0001) (**Fig. 3**). Peak *Cx. quinquefasciatus* capacity estimates occurred between June and August across all years and sites (mean: 0.76, range: 0.22 – 2.05) (**Fig. 2C and 2D**).

Empirical WNv infection

We found 1.9% (n = 242) of 12,912 mosquito pools positive for WNv (**S. Table 1**). *Culex quinquefasciatus* mosquitoes were confirmed as the primary epidemic vectors of WNv with 65.7% (n = 159) of WNv positive pools morphologically identified to *Cx. quinquefasciatus*. Unidentified *Culex* spp. mosquitoes, those that were damaged during collection unable to be morphologically assigned to a particular *Culex* species, accounted for 32.2% (n = 78) of WNv positive pools. Only 0.004% (n = 1) *Cx. restuans* samples tested positive for WNv; a sample

from IMP in July 2016. Additionally, 0.008% (n = 2) *Aedes albopictus* pools from GP in 2015 tested positive for WNv. Across all years and sites, detection of WNv in mosquitoes was limited to July to October with peak seasonal infection occurring in August 2014, July 2015, and August 2016 (**Fig. 3A**).

In total, 488 serum samples from 29 bird species were collected and tested for WNv antibodies (**S. Table 2**). 36.3% (n = 177) of all samples were serologically positive for WNv exposure. Samples from hatch year birds, birds born during the year of sampling, accounted for 32.2% (n = 157) of all samples with an overall antibody prevalence of 21.0% (n = 33). Detection of WNv antibodies in hatch year birds was limited to June through September with 51.5% (n = 17) of WNv positive hatch year samples collected in the month of August (**Fig. 3B**). Recaptured individuals accounted for 10.1% (n = 50) of all samples with 1 individual (0.02%) in GP sero-converting between August and September 2014. Only four viremic individuals were detected; all were sampled from GP in July and August across years.

There was no significant difference in the probability that a bird would test positive for WNv exposure between years (*GP data*: **Table 3, S. Table 3**) or between sites (*2016 data*: **Table 3, S. Table 3**). In both data sets, the probability that an individual tested positive for WNv exposure increased as an interaction between month and age (i.e. hatch year) (**Table 3, S. Table 3**) which most likely reflects the observed overall increase in WNv antibody prevalence in hatch year birds as each sampling season progressed (**Fig. 3B**). Importantly, the timing of avian WNv sero-incidence in hatch year birds, the sero-conversion in a recaptured individual in 2014, and the detection of WNv viremic individuals all corresponded to the timing and abundance of WNv infections in *Cx. quinquefasciatus* mosquitoes.

Discussion

All evidence of WNv transmission between mosquitoes and birds was statistically similar in sites with (GP) and without (IMP, SVP, P3) abundant, early season *Cx. restuans* populations. Peak *Cx. quinquefasciatus* abundance and vectorial capacity estimates closely corresponded to our field estimates of WNv transmission in the Atlanta region, supporting previous evidence that this species alone is the primary vector responsible for WNv enzootic transmission in the Atlanta region. Despite intense, early-season surveillance for WNv infected mosquitoes and birds in known hotspots for enzootic WNv transmission, we found no evidence of WNv transmission attributable to early season *Cx. restuans* populations. Additionally, our capacity estimates indicate that temperature-driven blood feeding and viral replication rates limit the likelihood that *Cx. restuans* could be an efficient and likely amplifying vector of WNv in temperate zones like Atlanta, GA. For vector species that reach peak abundance during inter-epidemic periods, i.e., time periods when susceptible host availability is low and temperatures are unfavorable for within-vector viral replication, it is most likely that these vector species only contribute a small, linear increase the prevalence of infections within host communities. Unless further research elucidates significant differences in the life history traits of *Cx. restuans* related to WNv transmission, it is unlikely that *Cx. restuans* significantly contributes to WNv simply by being an early season vector.

Our data provide further evidence that temperature and the availability of susceptible hosts are important determinants of vector-borne pathogen transmission. Previous WNv competence experiments have shown that extrinsic temperatures strongly influence the rate at which exposed vectors become infectious (27, 28). In the field, temperature has also been linked to increased prevalence of WNv infections in *Culex pipiens pipiens* mosquitoes (30). The

availability of susceptible hatch year birds has also been proposed as an important component of local WNv amplification (31); our empirical data supports this conclusion. Limited blood-feeding on WNv-competent hosts early in a transmission season by *Cx. restuans* populations has been proposed as a limitation of *Cx. restuans* as a vector of WNv (24). Our vectorial capacity model and WNv field surveillance comprehensively connect these results; long extrinsic incubation periods and the absence of WNv-susceptible hatch year birds strongly limit the likelihood that *Cx. restuans* is an efficient amplifying vector of WNv. Other mechanisms such as pathogen overwintering in primary vectors (32) or chronic infections in avian hosts (33) may be more important to the long-term persistence of vector-borne pathogens such as WNv during inter-epidemic periods.

It is possible that non-primary vector species can acquire infections in the field. For example, we detected one *Cx. restuans* and two *Ae. albopictus* samples that were WNv-infected; all three samples were collected during epizootic periods of WNv transmission. However, we posit that these infections are more likely due the abundance of infectious hosts generated by *Cx. quinquefasciatus* mosquitoes. Barnett (1960) lists four criteria for incriminating vectors as epidemiologically important: 1) vectors must feed on appropriate hosts for the pathogen, 2) there must be a spatial or temporal relationship between vector abundance and observed infections in hosts, 3) vector species must be commonly found infected in the field, and 4) the ability of the vector species to transmit the pathogen must be demonstrated, preferably experimentally (34). From our data, *Cx. restuans* did not share a temporal relationship with WNv antibody incidence in birds (criteria 2) nor did *Cx. restuans* samples test positive frequently for WNv infections (criteria 3). *Ae. albopictus* also did not frequently test positive for WNv infections (criteria 3) nor does this species preferentially blood feed on birds (criteria 1) (35).

Previous research on the ecology of *Cx. restuans* suggests that this species is more common in less human-mediated habitats such as wetlands (36). We cannot address the possibility that *Cx. restuans* is contributing more to WNv transmission in habitats in the southeast where it may be more common. However, in the Atlanta region our sample sites are confirmed hotspots of WNv transmission, so if *Cx. restuans* is more responsible for WNv transmission in other areas of Atlanta it has not led to an increase in the number of detected spatial hotspots (15, 16). Our assumption that catch basin collections approximate vector-host densities is an assumption that should be further examined. Changes in host abundance and species composition through time have been linked to differences in the timing of WNv infection in mosquitoes (37). Coupled with our assumption that feeding rates were random and thermally-driven, our vectorial capacity estimates may be under- or overestimated for *Cx. restuans* and *Cx. quinquefasciatus*. However, our analysis of minimum thermal and vector density thresholds indicates that for *Cx. restuans* to be an efficient, amplifying vector of WNv, regardless of habitat and host choice, *Cx. restuans* must either occur in extremely high abundance or bite hosts much more often than thermally predicted in order to overcome the limitations of early season extrinsic temperatures on within-vector WNv replication. Further research is needed regarding *Cx. restuans* life history traits relevant to WNv transmission and their relationship with temperature.

Secondary vector species in other pathogen systems can still be ecologically and epidemiologically relevant to transmission. However, our empirical results suggest that the role of these vectors in overall pathogen transmission is system-dependent and that transmission is not likely interdependent between vector species as suggested by recent theoretical models (6-8). In Kenya, successful malaria interventions that target contact rates between indoor blood-feeding, anthropophilic *An. gambiae s.s.* and human hosts has shifted the risk of malaria

transmission to outdoor blood-feeding, zoophillic *Anopheles arabiensis* Patton (38). Additionally, outbreaks of chikungunya (CHIKV) virus, primarily transmitted by *Aedes aegypti* Linnaeus have been attributed to secondary *Ae. albopictus* vectors in the French islands of Le Reunion (39) and Italy (40). Subsequent analyses of the circulating CHIKV strain in Le Reunion demonstrated that a mutation in the expression of a viral envelope protein increased infectivity and dissemination in *Ae. albopictus* individuals (41), indicating that it was a molecular change in the pathogen that expanded a vector species' transmission capabilities. Molecular incompatibilities between WNv and *Cx. restuans* have not been explored*;* however, recent studies show that rates of viral adaptation in mosquitoes are species-dependent (42). Previous studies of WNv evolution showed that the invasive NY99 strain was replaced by the WN02 strain which was more efficiently replicated in *Cx. pipiens* complex mosquitoes (43). Further research is needed to establish the likelihood of increased vector competence of secondary vector species due to genetic and molecular changes in circulating pathogens.

Greater considerations of extrinsic factors such temperature, host availability during inter-epidemic periods, and the molecular mechanisms behind vector expansion by pathogens are needed to better clarify under what circumstances non-epidemic vector species may impact pathogen transmission cycles. We recommend that future theoretical and empirical studies address these important ecological limitations when considering how and if observed patterns of vector-borne pathogen transmission are driven by a community of vectors rather than the actions of a primary vector.

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Table 1. Results from Poisson-error general linear mixed effect models for *Culex* spp. catch basin collections. For GP data, the reference year is 2014. For 2016 data, the reference site is GP. Month and Air temperature were centered to the median (July) and mean (22.93°C) values respectively in each data set.

Grant Park ONLY									
	Culex restuans			*Culex quinquefasciatus*			*Total Culex spp. collection*		
Variable	***Coeff.***	***S.E.***	**z-value**	***Coeff.***	***S.E.***	**z-value**	***Coeff.***	***S.E.***	**z-value**
Intercept	-0.44	0.18	-2.38*	1.54	0.13	12.1***	2.15	0.13	16.4***
Y:2015	-0.2	0.10	-1.88	-0.68	0.05	-12.8 ***	-0.55	0.04	-14.6***
Y: 2016	-0.04	0.10	0.42	-0.37	0.05	-7.95 ***	-0.41	0.03	-11.7***
Month	-0.43	0.02	-17.6***	0.08	0.01	7.64***	-0.07	0.01	-9.85***
Air Temp	0.01	0.01	0.72	0.1	0.01	19.1***	0.08	0.003	21.3***
Random Effect	**Var.**	**Std. Dev.**		**Var.**	**Std. Dev.**			**Var.**	**Std. Dev.**
Basin (n= 11)	0.29	0.54		0.16	0.40			0.18	0.42

2016 ONLY									
	Culex restuans			*Culex quinquefasciatus*			*Total Culex spp. collection*		
Variable	***Coeff.***	***S.E.***	**z-value**	***Coeff.***	***S.E.***	**z-value**	***Coeff.***	***S.E.***	**z-value**
Intercept	-1.92	0.23	-8.47***	1.27	0.24	5.3***	1.84	0.24	7.6***
IMP	-2.34	0.35	-6.61***	-0.27	0.36	-0.76	-0.47	0.37	-1.27
P3	-2.68	0.33	-8.16***	-1.29	0.35	-3.71***	-1.43	0.35	-4.09***
SVP	-2.89	0.39	-7.48***	-0.96	0.35	-2.72**	-1.18	0.36	-3.32***
Month	-0.47	0.07	-6.6***	0.16	0.01	16.3***	-0.06	0.01	8.08***
Air Temp	-0.08	0.03	-2.47*	0.04	0.004	8.32***	0.04	0.003	10.7***
Random Effect	**Var.**	**Std. Dev.**		**Var.**	**Std. Dev.**		**Var.**	**Std. Dev.**	
Basin (n= 11)	0.0	0.0		0.56	0.75		0.58	0.76	

*** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$

¶ Observation level random effect added to 2016 data model to improve convergence.

Table 2. Results from Gaussian-error linear mixed effects models for log+0.01 transformed *Culex* spp. capacity estimates. For GP data, the reference year is 2014. For 2016 data, the reference site is GP. Month and Air temperature were centered to the median (July) and mean (22.93°C) values respectively in each data set.

Grant Park ONLY									
	Culex restuans			*Culex quinquefasciatus*			*Total Capacity*		
Variable	***Coeff.***	***S.E.***	**t-value**	***Coeff.***	***S.E.***	**t-value**	***Coeff.***	***S.E.***	**t-value**
Intercept	-3.47	0.11	-30.7***	-1.73	0.19	-9.33 ***	-0.82	0.2	-4.18***
Y:2015	-0.35	0.13	-2.6**	-0.88	0.16	-5.44 ***	-0.81	0.15	-5.28***
Y: 2016	-0.34	0.13	-2.63**	0.03	0.16	0.22	-0.28	0.15	-1.89
Month	-0.26	0.02	-11.15 ***	0.14	0.03	5.15***	-0.04	0.03	-1.59
Air Temp	0.003	0.01	-0.31	0.23	0.01	17.0***	0.25	0.01	19.67***
Random Effect	**Int.**	**Res.**		**Int.**	**Res.**		**Int.**	**Res.**	
Basin (n= 11)	0.17	1.38		0.46	1.63		0.52	1.55	

2016 ONLY									
	Culex restuans			*Culex quinquefasciatus*			*Total Capacity*		
Variable	*Coeff.*	*S.E.*	t-value	*Coeff.*	*S.E.*	t-value	*Coeff.*	*S.E.*	t-value
Intercept	-3.78	0.06	-58.6 ***	-1.68	0.25	-6.84 ***	-1.08	0.30	-3.88***
IMP	-0.67	0.10	-6.77 ***	-0.75	0.37	-2.03	-0.95	0.42	-2.26*
P3	-0.78	0.10	-8.15 ***	-1.60	0.35	-4.54 ***	-1.95	0.40	-4.87***
SVP	-0.78	0.10	-7.97 ***	-1.17	0.36	-3.26**	-1.44	0.41	-3.53**
Month	-0.14	0.02	-8.52 ***	0.04	0.03	1.29	-0.05	0.03	-1.53
Air Temp	0.01	0.01	1.23	0.19	0.01	13.5***	0.23	0.01	16.6***
Random Effect	**Int.**	**Res.**		**Int.**	**Res.**		**Int.**	**Res.**	
Basin (n= 37)	0.12	0.93		0.72	1.66		0.83	1.64	

*** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$

Table 3. Odds ratio estimates for WNv antibody detection in birds from GP 2014 – 2016 and in all sampled sites in 2016. For GP data 2014 is the reference year and for 2016 data GP is the reference site.

	Variable	Estimate (95% CI)
Grant Park Only	Intercept	0.51 (0.16 – 1.14)
	Year: 2015	0.5 (0.23 – 1.06)
	Year: 2016	0.8 (0.4 – 1.6)
	Month	0.87 (0.72 – 1.04)
	Age: Hatch year	0.21 (0.09 – 0.42)*
	Month*Age (hatch year)	2.54 (1.55 – 4.4)*

	Variable	Estimate (95% CI)
2016 Only	Intercept	0.55 (0.23 – 1.13)
	Inman Park	0.7 (0.31 – 1.54)
	Phoenix Park	0.97 (0.39 – 2.36)
	Springvale Park	0.87 (0.39 – 1.93)
	Month	0.87 (0.72 – 1.06)
	Age: Hatch year	0.03 (0.006 – 0.1)*
	Month*Age (hatch year)	3.74 (1.78 – 8.84)*

* $p < 0.05$

Figure Legends

Figure 1. Contour plots for theoretical vectorial capacity estimates. Thick black lines with numbers represent capacity estimates. A) Vectorial capacity as a function of vector density and temperature, B) Vectorial capacity as a function of biting rate and temperature, C) Vectorial capacity as a function of survival and temperature. In B and C, vector density = 1.

Figure 2. Mean estimated vectorial capacity by month in GP (left-hand panels) and in all sites sampled in 2016 (right-hand panels). In each plot the horizontal dashed line designates Capacity = 1. A and B) total *Culex* spp. community capacity estimates, C and D) *Cx. quinquefasciatus* capacity estimates, E and F) *Cx. restuans* capacity estimates, G and H) unidentified *Culex* spp. capacity estimates. Dashed, colored lines in each plot represent the smoothed average capacity estimate for each plot.

Figure 3. West Nile virus (WNv) surveillance in Atlanta, GA 2014 – 2016. A) WNv minimum infection rate (MIR) estimates from all tested mosquitoes. MIRs are reported as the number infected mosquitoes per 1,000 individuals. B) The prevalence of WNv antibodies in sampled hatch year birds.

Figure 1.

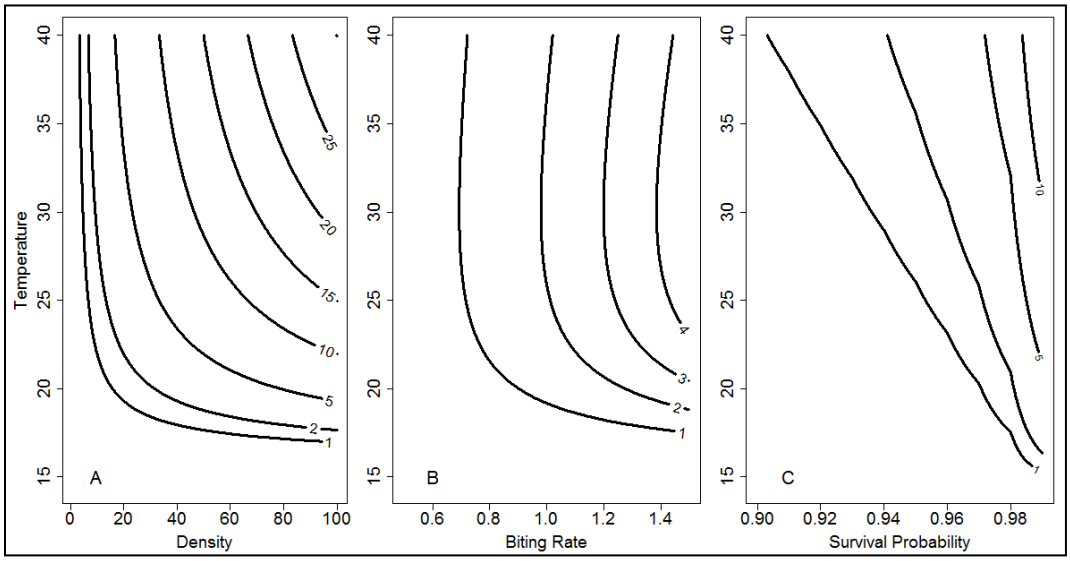

Temperature
Density
Biting Rate
Survival Probability
A
B
C
25
20
15
10
5
2
1
4
3
2
1
10
5
1

Figure 2.

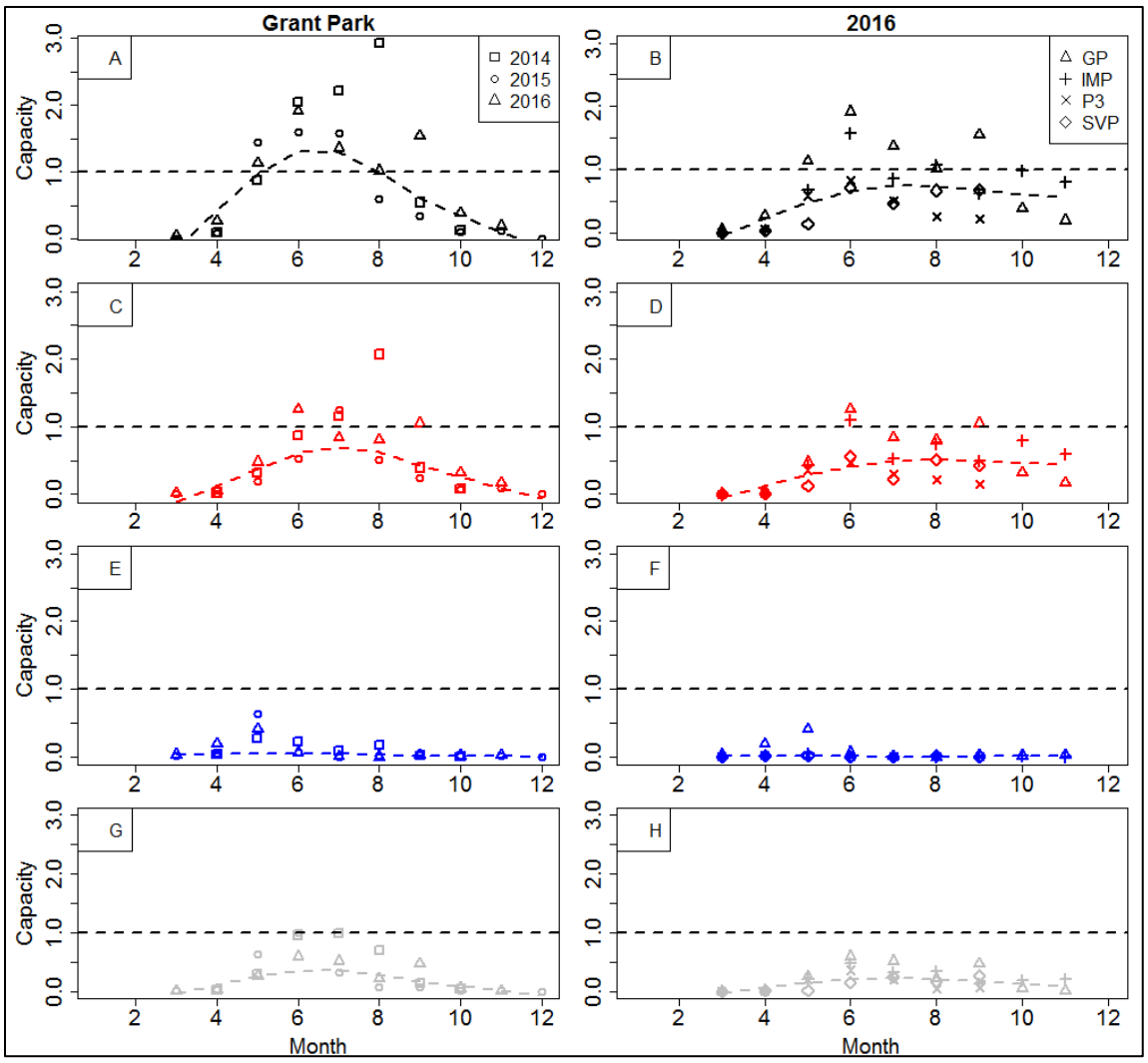
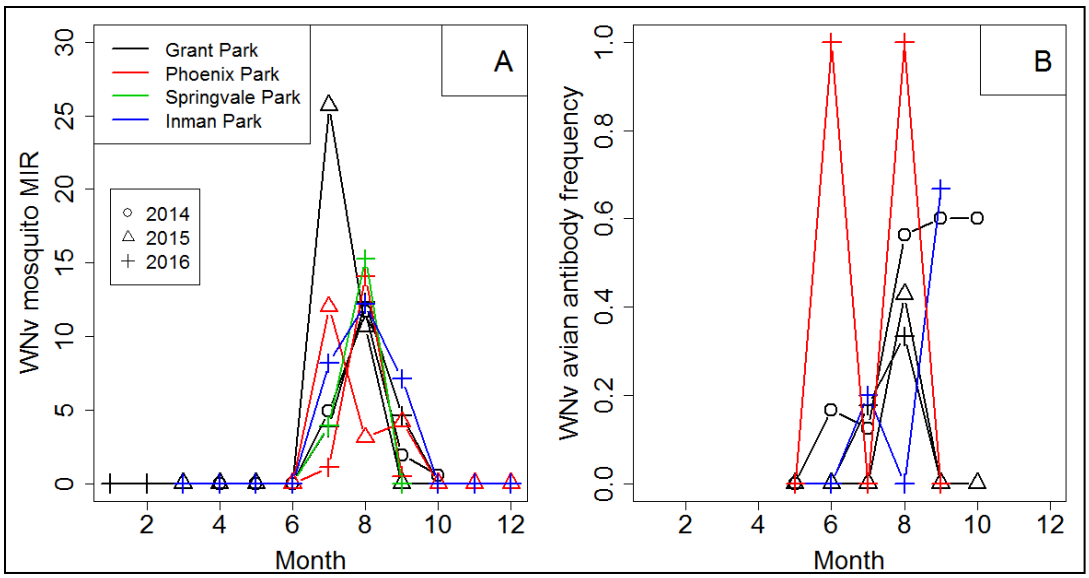

Grant Park
2016
A
B
C
D
E
F
G
H
2014
2015
2016
GP
IMP
P3
SVP
Capacity
Month
0.0
1.0
2.0
3.0
2
4
6
8
10
12

Figure 3.


A
B
Grant Park
Phoenix Park
Springvale Park
Inman Park
2014
2015
2016
WNv mosquito MIR
WNv avian antibody frequency
Month
Month
0
5
10
15
20
25
30
0.0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
12

Supplemental tables and figures

S. Table 1. Surveillance of West Nile virus infected mosquitoes from 2014 to 2016 in Atlanta, GA.

Year Sampled		Individuals collected (N pools)	Positive pools	MIR (95% CI)[1]
2014	Total	9,221 (1,073)	21	2.16 (1.37 – 3.24)
	Culex restuans	584 (121)	0	0
	Culex quinquefasciatus	3,468 (359)	11	3.24 (1.72 – 5.62)
	Culex spp.	5,424 (426)	10	1.87 (0.95 – 3.32)
	Other (n = 12 species)	410 (167)	0	0
2015	Total	14,213 (3,680)	56	3.86 (2.94 – 4.97)
	Cx. restuans	509 (117)	0	0
	Cx. quinquefasciatus	6,404 (572)	36	5.59 (3.99 – 7.63)
	Culex spp.	6,470 (2,747)	18	2.69 (1.65 – 4.45)
	Other (n = 13 species)	830 (244)	2	2.36 (0.42 – 7.69)
2016	Total	47,984 (8,159)	162	3.48 (2.9 – 4.04)
	Cx. restuans	2,957 (426)	1	0.34 (0.01 – 1.68)
	Cx. quinquefasciatus	21,166 (1,833)	112	5.62 (4.67 – 6.70)
	Culex spp.	23,172 (5,552)	50	2.16 (1.63 – 2.81)
	Other (n = 10 species)	913 (347)	0	0
Total	Total	71,418 (12,912)	242	3.51 (3.07 – 3.99)
	Cx. restuans	4,050 (664)	1	0.35 (0.02 – 1.71)
	Cx. quinquefasciatus	31,038 (2,764)	159	5.12 (4.34 – 6.01)
	Culex spp.	42,932 (8,725)	78	2.51 (2.00 – 3.11)
	Other (n = 15 species)	2,153 (758)	2	0.93 (0.17 – 3.03)

[1] MIR values are listed as the total minimum infection rate for the year of collections. Temporal trends in MIR values are displayed in Figure 3.

S. Table 2. . Surveillance of WNv antibody prevalence in sampled bird communities in Atlanta, GA 2014 – 2016.

Year Sampled	Species	N (% total)	WNv + N (% total)	Hatch years N (% total)	WNv + N (% HY)	Viremic N (% total) Age/Species/Date
2014	**Total**	**101**	**42 (41.6)**	**41 (40.6)**	**17 (41.5)**	**1 (0.01)**
	American Robin	6 (5.9)	1 (16.7)	4 (66.7)	1 (25)	0
	Northern Cardinal	10 (9.9)	7 (70)	1 (10)	1 (100)	0
	Mimids¶ (n = 3 species)	51 (60.4)	25 (49)	24 (47.1)	14 (58.3)	GRCA: 07/22
	Other (n = 11 species)	34 (33.7)	9 (26.5)	12 (35.3)	1 (8.3)	0
2015	**Total**	**85**	**23 (27.1)**	**25 (29.4)**	**4 (16)**	**2 (0.02)**
	American Robin	19 (22.4)	5 (26.3)	8 (42.1)	1 (12.5)	HY*: 07/21
	Northern Cardinal	7 (8.2)	4 (57.1)	3 (42.9)	2 (66.7)	0
	Mimids¶ (n = 3 species)	24 (28.2)	9 (37.5)	5 (20.1)	1 (20)	HY* NOMO: 08/04
	Other (n = 15 species)	35 (41.2)	5 (14.3)	9 (25.7)	0	0
2016	**Total**	**302**	**112 (37.1)**	**91 (30.1)**	**12 (13.2)**	**1 (0.003)**
	American Robin	90 (29.8)	32 (35.6)	29 (32.2)	4 (13.8)	AHY**: 08/12
	Northern Cardinal	50 (16.6)	34 (68)	14 (28)	4 (28.6)	0
	Mimids¶ (n = 3 species)	73 (24.2)	27 (37)	33 (45.2)	3 (9.1)	0
	Other (20 spp.)	89 (29.5)	19 (21.3)	15 (16.9)	1 (6.7)	0
All years	**Total**	**488**	**177 (36.3)**	**157 (32.2)**	**33 (21)**	**4**
	American Robin	115 (23.6)	38 (33)	41 (35.7)	6 (14.6)	2
	Northern Cardinal	67 (13.7)	45 (67.2)	18 (26.9)	6 (33.3)	0
	Mimids¶(n = 3 species)	148 (30.3)	61 (41.2)	62 (41.9)	18 (29)	2
	Other (n = 25 species)	158 (32.4)	33 (20.9)	36 (22.8)	2 (5.6)	0

¶ Mimids include Brown Thrashers, Gray Catbirds (GRCA), and Northern Mockingbirds (NOMO).
* HY = hatch year
** AHY = after hatch year

S. Table 3. Results from binomial-errors GLMMs for WNv antibody prevalence in Grant Park 2014 – 2016 only and 2016 sampled sites only. In each model month is centered to July.

	Variable	***Coefficient***	***Standard Error***	***Z-value***
Grant Park ONLY	Intercept	-0.67	0.45	-1.48
	Y: 2015	-0.7	0.39	-1.81
	Y: 2016	-0.22	0.35	-0.63
	Month	-0.14	0.09	-1.51
	Age: HY	-1.58	0.38	-4.17***
	Month*Age: HY	0.93	0.26	3.56***
	Random Effect	Variance	Std. Dev.	
	Species (n=24)	1.34	1.16	
	Variable	***Coefficient***	***Standard Error***	***Z-value***
2016 ONLY	Intercept	--0.59	0.39	-1.53
	Inman Park	-0.36	0.4	-0.88
	Phoenix Park	-0.03	0.45	-0.08
	Springvale Park	-0.14	0.4	-0.34
	Month	-0.14	0.1	-1.4
	Age: HY	-3.53	0.73	-4.84***
	Month*Age: HY	1.32	0.4	2.28***
		Variance	Std. Dev.	
	Random Effect Species (n=25)	1.18	1.09	

******* $p < 0.001$

Figure Legends

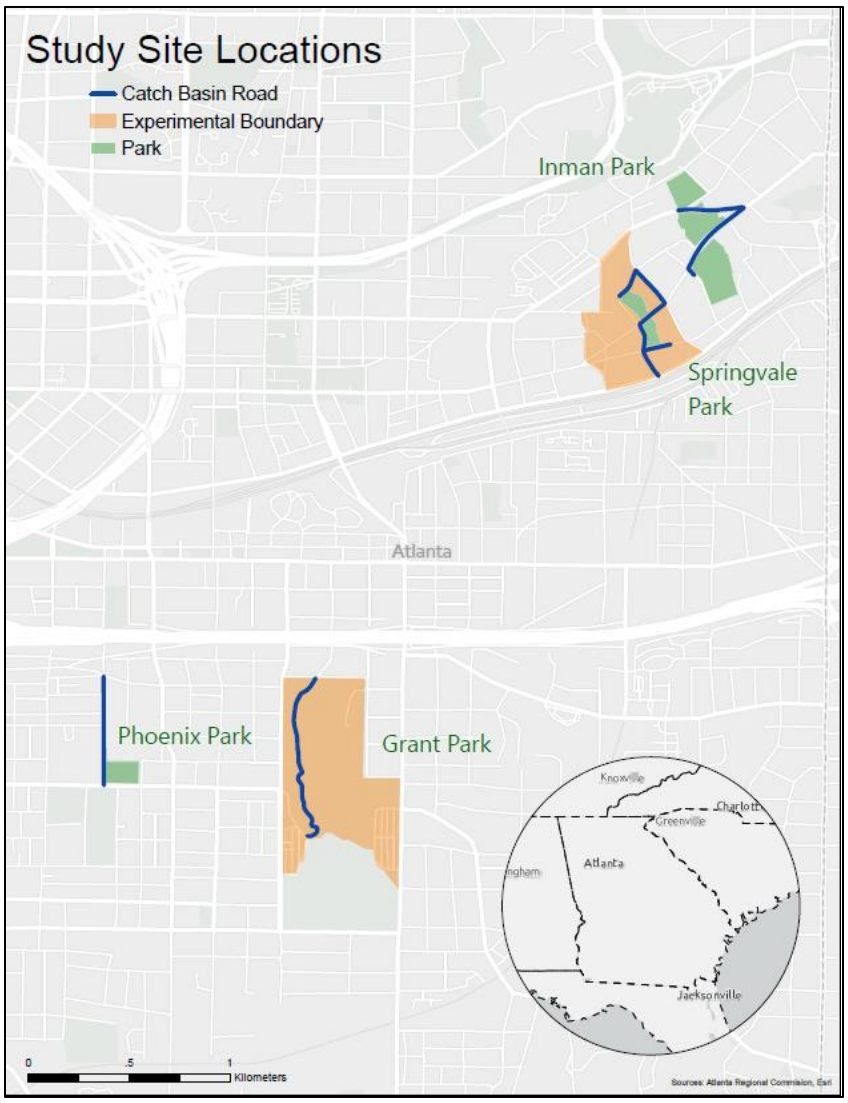
S. Figure 1. Map of Atlanta, GA and location of the four sample sites.

S. Figure 2. Scatter plot of 1/median survival times from Ciota et. al. 2014 with fitted mortality rate line. Dotted line and crosses represents *Cx. restuans* data; dashed line and triangles represents *Cx. quinquefasciatus* data; solid line and open circles represents *Cx. pipiens pipiens* data; heavy, solid line is line through all data.

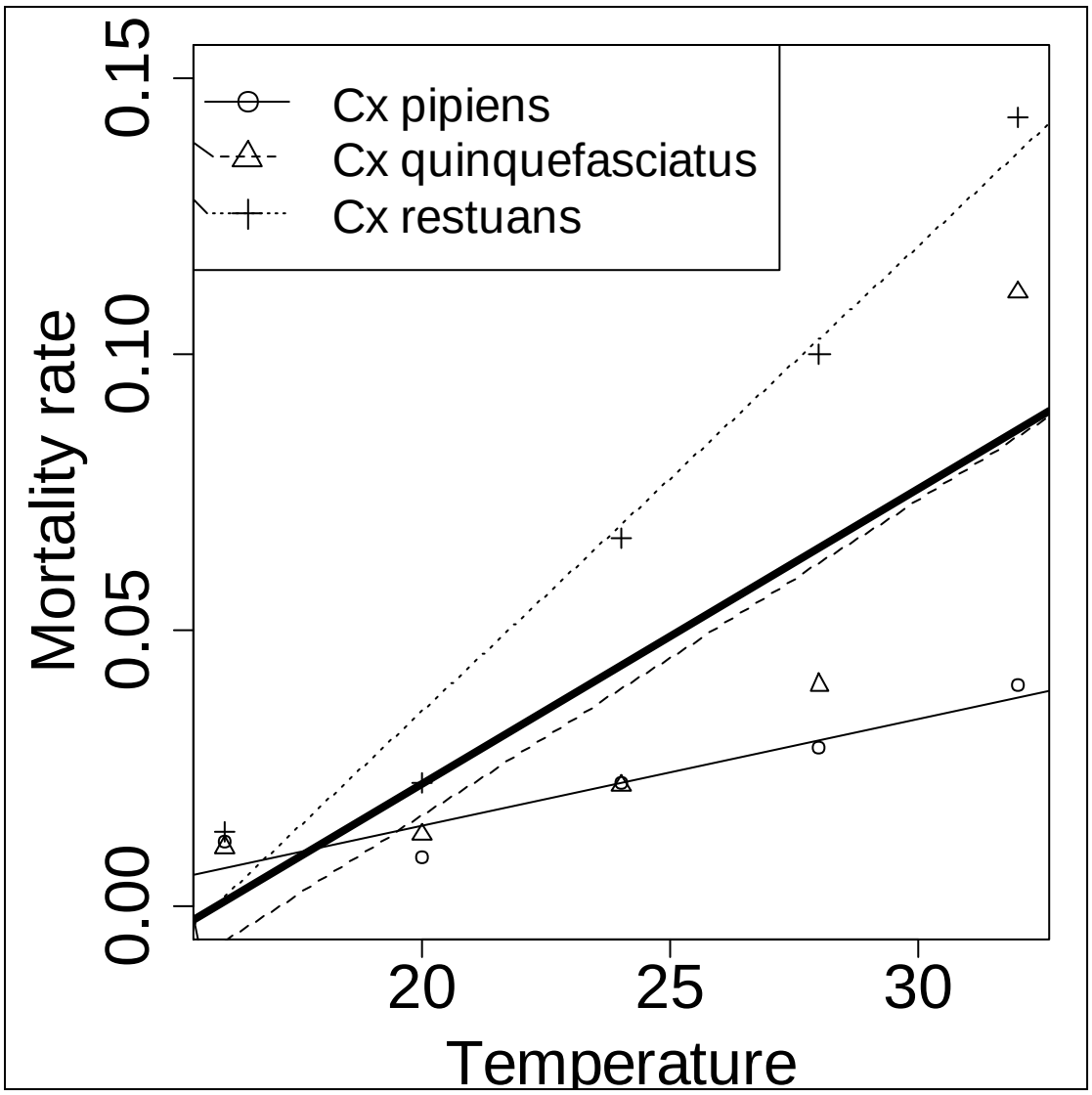
S. Figure 3. Panel plot of A) average daily temperature in Atlanta, GA 2014-2016 by week and B) predicted biting rate, C) predicted surival probabilities, and D) predicted length of the WNv extrinsice incubation period by week in Atlanta, GA.

S. Figure 4. Observed *Culex* spp. catch basin collections in Grant Park (left panels) and in 2016 (right panels). Blue boxplots represent *Cx.* restuans collections (A and B), red boxplots represent *Cx quinquefasciatus* collections (C and D), grey boxplots represent unidentified *Culex* spp. collections (E an F), and white boxplots represent total *Culex* spp. catch basin collections (G and H).

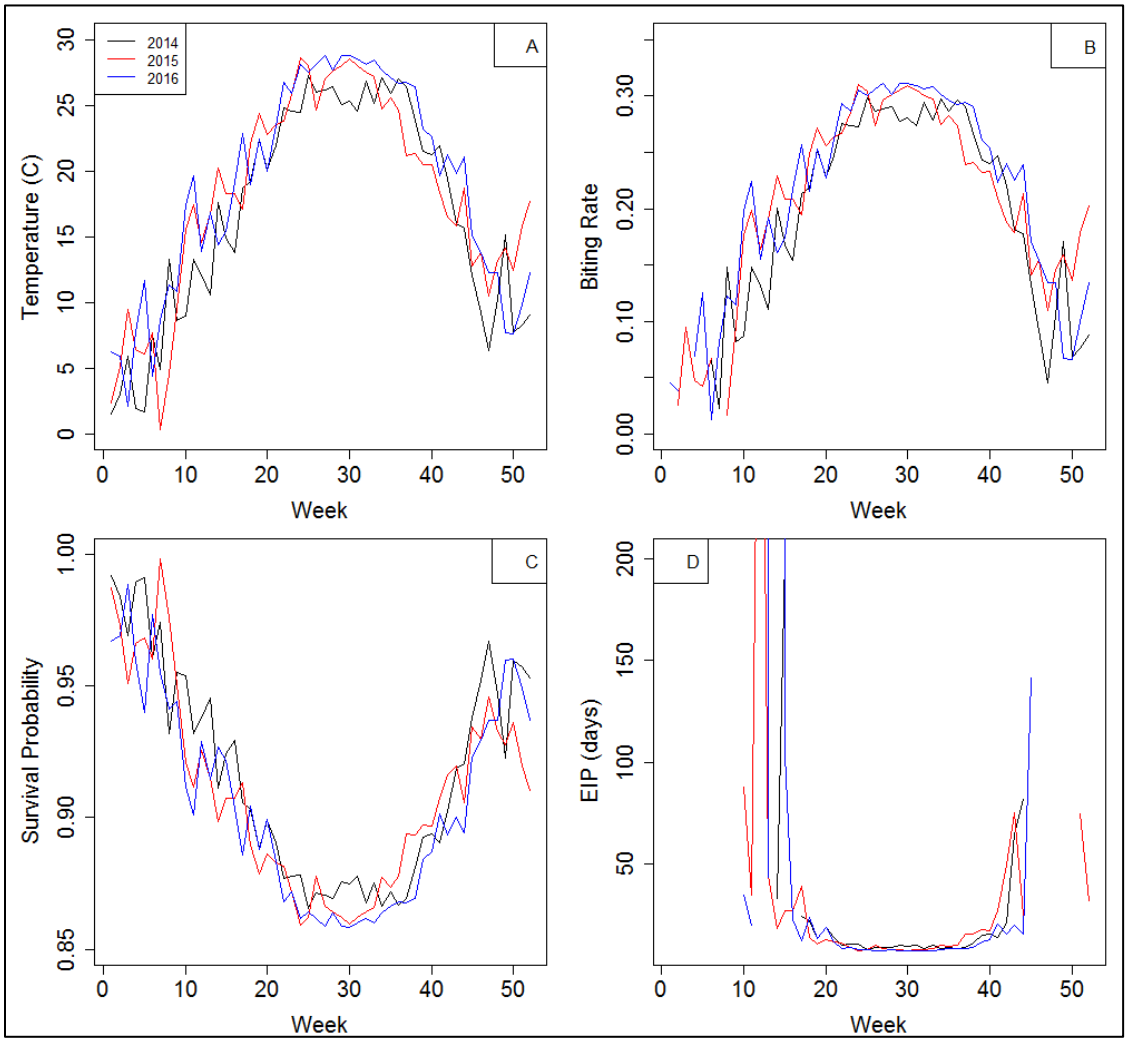
S. Figure 1.


Study Site Locations
Catch Basin Road
Experimental Boundary
Park
Inman Park
Springvale Park
Atlanta
Phoenix Park
Grant Park
Knoxville
Charlotte
Greenville
Atlanta
Jacksonville
0
.5
1
Kilometers
Sources: Atlanta Regional Commission, Esri

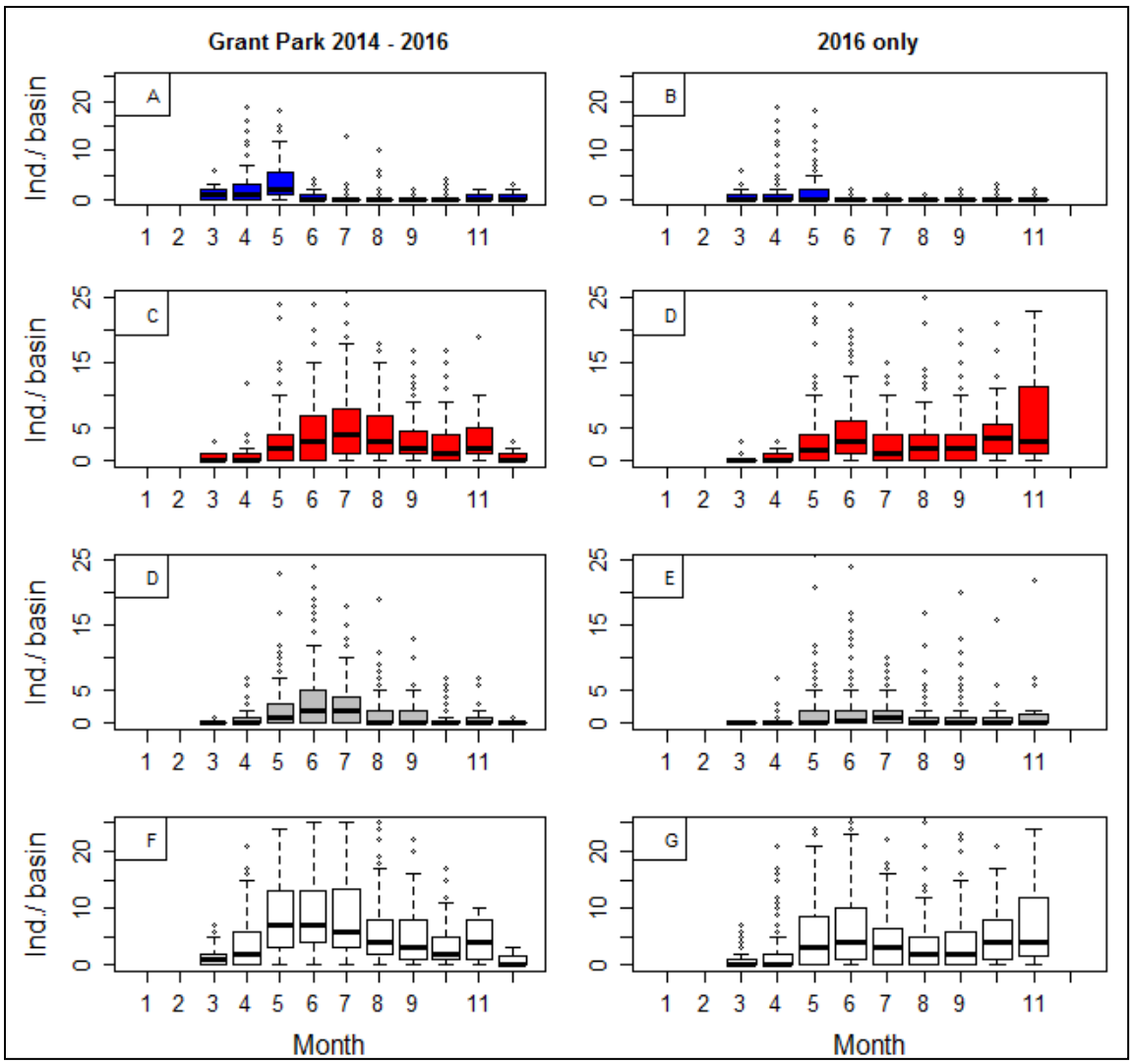
S. Figure 2.


Cx pipiens
Cx quinquefasciatus
Cx restuans
Mortality rate
0.00
0.05
0.10
0.15
20
25
30
Temperature

S. Figure 3.


2014
2015
2016
A
Temperature (C)
Week
B
Biting Rate
Week
C
Survival Probability
Week
D
EIP (days)
Week

S. Figure 4.



Chapter 2: Species-varying cumulative force of infection for West Nile virus

To be published with the following authors: Joseph R. McMillan, Gabriel Hamer, Rebecca Levine, Daniel Mead, Lance A. Waller, Tony Goldberg, Edward Walker, Jeffery Brawn, Marilyn Ruiz, Uriel Kitron, Gonzalo Vazquez-Prokopec

Introduction

Heterogeneity, i.e. biologically significant variation, is a pervasive attribute of most host-parasite systems (1), most often evidenced in spatio-temporal aggregations of infections (2), non-random host-pathogen contacts (3), or individual as well as population level variation in infectivity (4, 5). Quantifying sources of variability can lead to hypotheses regarding the underlying processes that drive observed patterns of infection. For instance, it is a common observation in vector-borne disease systems that vector blood meal sources aggregate on particular host individuals or species (6-8). Theoretical models of transmission that account for heterogeneous vector-host contacts predict elevated rates of pathogen transmission compared to random blood feeding events (3, 9-11). In terms of the control of vector-borne diseases, whom or what vectors feed on has large implications for the control and prevention of transmission among humans or between wildlife reservoirs and human populations (12).

In wildlife systems, vectors feed on a diverse assemblage of species, and patterns of vector-host contacts are most commonly estimated using molecular blood meal identification of field collected vectors (13); vector-host preferences are then quantified by weighing the relative proportion of blood meals identified from a particular host species to that host species' index of relative abundance in the field (14). Coupled with indexes of host pathogen competence which define the infectiousness of a host to biting mosquitoes (15), vector host choice studies have been

the primary approach employed throughout the United States to evaluate the host species most important to the transmission dynamics of West Nile virus (WNv) (16-18).

West Nile virus is a zoonotic arbovirus mainly transmitted by *Culex* spp. mosquitoes (primarily members of the *Culex pipiens* complex) among birds. Direct empirical evidence of transmission in hosts is limited by the short duration of an infectious viremia for many viral pathogens such as WNv (19, 20). Instead, estimates of WNv exposure (i.e., the presence of neutralizing antibodies) are used to indirectly quantify variability of WNv transmission in host populations. Broadly, WNv or antibodies to WNv have been detected in over 300 native and introduced bird species in the U.S. (21), and serosurveys show heterogeneous patterns of WNv exposure in bird communities across the country (19, 20, 22-26). However, serosurveys often lack temporal replication, such that these studies provide only brief glimpses into the process of WNv transmission in space and time. These short sampling periods also limit the sampling og hatch year (i.e. juvenile) birds, which are susceptible to WNv within a few days to weeks after hatching as maternally acquired antibodies decay (27, 28). Quantifying the incidence of WNv antibodies in hatch year birds across space and time could provide indirect measures of the magnitude of the enzootic cycle and the force of infection (FOI), i.e., the rate at which new cases arise, of WNv in host communities.

Accurate FOI estimates rely on knowing the specific age of an individual and the timing of infection (29, 30). These two quantities are difficult to estimate in serosurveys of wild birds, yet each can be approximated. Age in wild bird communities is classified as a stage, and while the specific age of a bird is unknown birds can be accurately classified as hatch year birds. Although serosurveys cannot identify the timing of WNv exposure, by focusing analyses on hatch year birds one can attribute incidence to a specific year.

We capitalized on an unprecedented dataset from two long-term serosurveys of WNv in avian populations from Chicago, IL, U.S. and Atlanta, GA, U.S., in order to quantify variability in WNv transmission across years and host species. Previous findings regarding WNv exposure in avian communities from these two cities have been reported elsewhere (18-20, 31-36). *A priori* we predicted that seroincidence estimates would vary annually within in each city; however, we did not expect to see any significant differences in WNv seroincidence for resident species, i.e. non-migratory, between cities. Of particular interest was the comparison of species-level seroincidence estimates. Given results from previous *Culex pipiens* complex blood meal studies in each city that identified American robins in Chicago and American robins and northern cardinals in Atlanta as important blood meal hosts (18, 20, 31), we hypothesized that American robins (*Turdus migratorius*) would show the greatest evidence of WNv exposure in bird communities in Chicago and that northern cardinals (*Cardinalis cardinalis*) would show the greatest evidence of WNv exposure in Atlanta. Our analyses present empirical evidence of heterogeneous WNv exposure in wild bird communities that are consistent with both community-level and species-level contributions to pathogen transmission.

Materials and Methods

Avian WNv surveillance methodology

In both Chicago, IL and Atlanta, GA, surveillance sites were chosen to represent a range of microhabitats commonly utilized by *Culex* spp. vectors and avian species in urban environments (**S. Fig. 1**). In each city, avian communities were sampled weekly from May to October to monitor WNv antibody serostatus, an indicator of prior WNv exposure. Birds were captured using mist nets following protocols developed by (19, 20). Mist netting took place between dawn (approx. 30 mins prior to sunrise) and 1300 hrs in the absence of precipitation and

high wind speeds (> 15 km/h). Captured individuals were identified to age, sex, and species, and all individuals were given a unique coded aluminum or steel band following (37). Up to 200 µl of blood was collected via jugular venipuncture from birds weighing approximately > 10 g and in suitable physical condition (e.g., no injuries or signs of severe stress). Blood samples were held on ice in the field. In the laboratory, blood samples were centrifuged for 10 min at 10,000 rpm, and serum was separated from blood clots using sterile pipettes. Sera were stored at -80°C until serological tests could be performed.

All sera were tested for IgY (an avian immunoglobulin functionally similar to the mammalian IgG) antibodies; all sera from Chicago, IL and Atlanta, GA 2010 – 2013 were tested using epitope-blocked enzyme-linked immunosorbent assay (ELISA) techniques following (19) (see also (36)) while sera from Atlanta, GA 2014 - 2016 were tested using serum neutralization tests (SNT) at the University of Georgia's Veterinary Diagnostic Laboratory (Tifont, GA) following Chapter 2.1.24 of the OIE Diagnostic manual (38). Though different methods were used to determine antibody presence, previous studies have reported similar results for ELISA and PRNT methods (36). Additionally, a subset of samples from Atlanta 2010 – 2013 (tested using blocking ELISA) were screened at the veterinary clinic (the clinic was not informed as to which samples were negative or positive) to determine if SNT results could confirm ELISA results; using this blind testing approach, SNT successfully determined ELISA positive and negative results.

ELISA methods

In brief, the assay consisted of a monoclonal capture antibody, a WNV recombinant antigen, a labeled monoclonal antibody, and avian serum. After multiple incubations and washes,

the reduction in optical density was determined and percent inhibition calculated: percent inhibitions equal to or greater than 60% were considered WNv positive. All sera were initially screened at a dilution of 1:20. Samples testing positive in the initial screening were serially diluted (up to 1:640) and rescreened to confirm results and determine endpoint titers.

SNT methods

All sera were heat inactivated for 30 minutes at 56°C and then serially diluted, starting at 1:4 - 1:4096 in cell-culture media. First equal parts virus (100-300 $TCID_{50}$ per 1 ml) and serum (50µl each) was added to the culture and incubated at 37°C. After one-hour incubation, 100ul of cells were added and plates were incubated at 37°C for 3-5 days and read for cytopathic effects (CPE). Endpoint titres were based on the last well to display complete protection against CPE. Negative and positive controls were run as well as a cell control and virus back titration control to ensure testing integrity. Titers equal to or greater than 1:8 were considered antibody positive.

For the purposes of our analyses, individuals testing positive for WNv exposure per the above the thresholds were labeled as 1 – WNv Positive and 0 – WNv negative.

Data analysis

The presence or absence of WNv antibodies in a sampled hatch year individual (i.e., serostatus at the individual level and seroincidence at the population level) was chosen as the primary unit for evaluating patterns of WNv transmission because hatch year birds are born effectively susceptible to WNv (27). Thus, seroinicidence estimates can be used to approximate the epidemiological force of infection, or rate at which susceptible individuals acquire infection, experienced by resident avian communities (39, 40). Our analyses first focused on identifying

variation in WNv seroincidence between years and species in each city independently. We then compare WNv seroincidence between both cities for the period both datasets overlapped (2010 - 2012). Generalized linear mixed-effects models (GLMMs) were used to analyze all data due to potential positive correlations between repeated spatial and temporal measurements. To reduce pseudo-replication, only the first sample from a captured individual was included in each analysis. Additionally, data was not included for sampled individuals that could not be identified to age, species, or for which the location or date of sampling was unspecified. To improve model convergence, the variable ‘’Week’ was centered to the median value in the data set. Following prior findings (20), sex was not included in any model.

Analyses comparing incidence by year for each city relied on binomial-error GLMMs with Week and Year of sampling as fixed effects and ‘Species’ as a random effect. Preliminary analyses revealed almost zero variation in serostatus between replicated sample sites within each city. Additionally, there were no differences by AIC between models that did or did not contain location of sampling within each city as a random effect; all models preceded with only ‘Species’ as a random effect. Treating ‘Species’ as a random effect allowed us to make predictions regarding host variability in the contribution to WNv. In all GLMMs, ‘2010’ was the reference variable for Year. In GLMMs of Atlanta, preliminary analyses showed there was no difference in WNv antibody detection between Method (ELISA vs. SNT) (Method: SNT, $p > 0.3$, $\Delta AIC < 1$), so ‘Method’ was not included in models of comparing WNv seroincidence.

For analyses comparing WNv seroincidence between cities, preliminary analyses predicted zero variance by sample location, so we utilized a binomial general linear mixed effect model with Week, Year, and City as fixed effects. Because Species codes were similar between cities, we modeled Species as a nested random effect within City. In these models, ‘2010’ and

'Atlanta' were the reference variables for fixed categorical effects. To further refine our comparisons of enzootic transmission between cities, we restricted our analyses to data collected during WNv epidemic periods which we defined as MMWR week 24 to 40.

All GLMMs were implemented in R using the 'lme4' package (41). Serostatus predictions were generated from each GLMM with confidence intervals generated by boostrapping the data with the bootMER function. Odds ratios plots for random effects as well as marginal effects plots for fixed effects were generated using the sjPlot package in R (42). All other analyses also took place in R (43).

Results

Observed West Nile virus seroprevalence

In total, 6,649 unique samples from 94 species were collected between Chicago, IL (82 spp., n = 5,564) and Atlanta, GA (45 spp., n = 1,085). House sparrows and American robins accounted for over 50% of collected samples in Chicago while American robins, northern cardinals, and northern mockingbirds accounted for 50% of collected samples in Atlanta (**S. Table 1**). In Chicago, evidence of WNv exposure was detected in 27 species with an overall seroprevalence of 10.0%. WNv seroprevalence among the 10 most commonly sampled species in Chicago varied from 0.7% in Swainson's thrushes to 47.0% in northern cardinals (**S. Table 1**). In Atlanta, evidence of WNv exposure was detected in 19 species with an overall seroprevalence of 30.0% (**S. Table 1**). WNv seroprevalence estimates among the 10 most commonly sampled species in Atlanta varied from 2.5% in European starlings to 53.7% in northern cardinals (**S. Table 1**). In general, WNv seroprevalence estimates across sampled species were higher in Atlanta compared to Chicago (**S. Table 1**).

Observed West Nile virus seroincidence

In total, 2,814 samples from 63 species (Chicago: 54 spp., n = 2,580; Atlanta: 26 spp., n = 324) were collected from hatch year individuals (**Table 1**). In Chicago, the overall WNv seroincidence estimate was 7.3%. House sparrows and American robins were the most commonly sampled hatch year species in Chicago (**Table 1**), and across years, American robins were commonly sampled in early summer (May and June) while House sparrows were commonly sampled during the primary WNv epidemic months (July through October) (**S. Fig. 2**). Overall, seroincidence in the most commonly sampled hatch year species in Chicago varied from 0% in white-throated sparrows to 36.1% in northern cardinals. Though northern cardinals had the highest observed seroincidence among all sampled species in Chicago, northern cardinals never comprised more than 10%, on average, of any monthly sample size.

In Atlanta, overall WNv seroincidence was 18.2% (**Table 1**). Similar to Chicago, American robins were the most commonly sampled hatch year species in early summer (**S. Fig. 3**), while American robins, northern cardinals, and northern mockingbirds were average equally sampled during July and August; during this time period each species accounted for approximately 20% of the sample (**S. Fig. 3**). Seroincidence in the most commonly sampled species varied from 0.0% in brown thrashers, eastern towhees and song sparrows and 40.5% in northern mockingbirds.

Comparisons of WNv seroincidence within each city

All GLMMs predicted that the probability an individual would test positive for WNv rose exponentially across all sampled species during a transmission season. In Chicago, the marginal effect of Week had an approximate predicted probability rising from approximately 0% in week 21 to approximately 8% in week 44 (**S. Fig. 4**). In Atlanta, the marginal effect of Week was

much more pronounced with a predicted probability of 0% in week 19 which rose to approximately 60% in week 45 (**S. Fig. 5**). Across all sampled species in Chicago, the probability of a hatch year individual testing positive for WNv varied significantly by Year, and seroincidence of WNv in wild birds was significantly elevated in 2005 and 2012 compared to the reference year, 2010 (**Fig. 1, S. Table 2**); seroincidence estimates were also significantly depressed in 2007 compared to the reference year, 2010. There were no significant differences in WNv seroincidence rates between Years in Atlanta (**Fig. 2, S. Table 3**).

The predicted probability of a hatch year testing positive for WNv varied significantly among sampled hatch year species (**Fig. 3 and 4**). In both cities, GLMMs determined that northern cardinals had the highest predicted probability of testing positive for WNv across all species (**Fig. 3 and 4**), and that this probability was significantly different from American robins (**S. Fig. 6 and 7)**. In addition to northern cardinals in Chicago, the odds of testing positive for WNv were also significantly greater than 1 for house sparrows, red-winged blackbirds, American robins, gray catbirds, house finches, European starlings, blue jays, brown-headed cowbirds, and mourning doves (**S. Fig. 6**). In Chicago, the odds of seroincidence was significantly less than one for white-throated sparrows (**S. Fig. 6**). Comparing 95% confidence limits of estimates, seroincidence was significantly higher in northern cardinals compared to American robins, gray catbirds, house finches, and house sparrows (**S. Fig. 6**)**.** In addition to northern cardinals in Atlanta, the odds of testing positive for WNv were also significantly greater than 1 for northern mockingbirds (**S. Fig. 7**); however, all confidence limits for WNv seroincidence in each species overlapped.

Comparisons of WNv seroincidence between cities

In GLMMs comparing WNv seroincidence between Atlanta, GA and Chicago, IL, City had a small but significant marginal effect on the predicted probability of an individual testing positive for WNv (**S. Table 4, S. Fig. 8**), and the probability of an individual testing positive for WNv was higher in Atlanta than in Chicago. The marginal effect of Week in both cities predicted approximately a 0% probability of an individual testing WNv positive in week 24 and approximately 20% in week 40 (**S. Fig. 8**). WNv incidence varied significantly by year in both cities: the probability an individual tested positive was higher in 2012 compared to 2010 (**Table 3, Fig. 5, S. Table 4**). Across all sampled species, predicted probabilities were highest for northern cardinals in Atlanta and Chicago, with northern mockingbirds in Atlanta, and house sparrows, gray catbirds, and American robins in Chicago also having a significant chance of testing positive for WNv (**Fig. 6, S. Fig. 9**). In this analysis, all 95% confidence intervals overlapped among odds ratio estimates for species-specific WNv seroincidence.

Discussion

Our results identify heterogeneities of WNv exposure in host communities consistent with both community-level as well as species-level contributions to transmission. In general, WNv seroincidence varied by year, species, and location. General linear mixed effects models predicted that the probability of a hatch year individual testing positive for WNv was generally higher across all sampled species in Atlanta than Chicago. This is an unexpected result given previously published WNv minimum infection rates in mosquitoes as well as reports of human WNv neuroinvasive disease in Chicago compared to Atlanta (19, 20, 44-46). Additionally, species-specific predicted probabilities of seroincidence were highest in northern cardinals in both Atlanta and Chicago; WNv incidence curves were statistically similar for this species in

GLMMs specifically comparing Cities. This result is also unexpected given previous reports that American robins are preferred blood meal hosts of *Culex pipiens* complex mosquitoes in Chicago and the primary amplifying host species of WNv in each city (18, 20, 47).

Our data show that the abundance of hatch year birds increased throughout a sampling season, and the likelihood of a hatch year testing positive for WNv accumulated as a transmission season progressed. Thus, our GLMM predictions could be termed the cumulative FOI. We believe this metric approximates the epidemiological force of infection, which is the rate at which new cases arise in the population, for WNv in host communities. Our analyses predicted that both the odds of testing positive for WNv and the cumulative FOI of a given species varied significantly among species. In both cities, northern cardinals had the highest probability of encountering WNv throughout a transmission season. In Chicago, the odds of WNv exposure were also significant for other species such as American robins and house sparrows, which have previously been proposed as the primary amplifying hosts of WNv in the city (18). In Atlanta, northern mockingbirds experienced a statistically similar cumulative FOI to northern cardinals.

Previous investigations of mosquito blood feeding and WNv transmission in each city have generated predictions as to which host species are most important to WNv. In Chicago, American robins, blue jays, and house finches were predicted to generate the greatest number of infectious mosquitoes (31), and American robins were additionally predicted to be the primary driver of the force of WNv infections in mosquitoes at local scales (18). In Atlanta, high seroprevalence rates in northern cardinals and members of the Mimidae family were attributed to blood feeding shifts away from American robins, which may amplify the virus early in a transmission season, on to cardinals and mimids during WNv epidemic periods (20). *Culex* spp.

feeding shifts onto low to moderately competent hosts in Atlanta has additionally been proposed as a possible mechanism for reduced spillover of WNv in humans in Atlanta (20). Feeding shifts away from American robins have not been identified in the Chicago data set, and declining feeding rates on American robins was linked to declining abundance of American robins as a season progressed (31). Because we do not have blood feeding data that corresponds to all years of avian WNv sampling, further study is needed to determine why the pattern of increased incidence in northern cardinals compared to American robins is strikingly similar between cities despite different proposed mechanisms of amplification in the WNv system.

High WNv seroprevalence estimates have been previously reported for northern cardinals in numerous sites throughout the country (20, 24-26, 33, 48-50), and previous serosurveys from both Georgia and Illinois have proposed northern cardinals as a sentinel species of local WNv transmission (22, 25, 33). Our results extend these previous findings by demonstrating that WNv seroincidence across years was significantly higher in this species compared to others. The high predicted probability of WNv exposure in northern cardinals possibly suggests a greater role for this species in WNv transmission cycles in the U.S. Previous theoretical studies have identified American robins as the primary amplifying host species of WNv in the mid-Atlantic, northeastern, and midwestern regions (16-18); however, our cumulative FOI estimates were highest in other locally abundant host species. Why incidence is highest in northern cardinals warrants further study. Because northern cardinals are widely distributed across the midwestern and eastern U.S., per capita WNv exposure in northern cardinal populations may be higher than in American robins. Because robins often aggregate in roosts, roosting behaviors have been experimentally shown to reduce per capita vector host biting rates and incidence of WNv (51).

There may also be differences in species-specific demographics such as brood size and nestling mortality that bias our detection of WNv seroincidence patterns.

Our use of serological methods to identify patterns of WNv incidence has its limitations. Our seroincidence estimates are biased towards ground-dwelling avian species and are subject to issues of sample size. Additionally, antibody presence cannot define the timing of pathogen exposure nor is it indicative of the amount of virus the host was initially exposed to or produced during infectious periods (47). However, we limited our analyses to seroincidence in hatch year birds which are susceptible to WNv infections after fledging and leaving the nest (27). In general, antibody presence within hatch year individuals indicates that transmission occurred within the year, and thus it can provide an indirect measure of the intensity of WNv enzootic activity. Unobserved field mortality (due to infection, predation, competition, or chance) could partially explain the low prevalence of WNv antibodies in American robins as well as other species (47, 52). West Nile virus challenge studies in seronegative adult American robins note a dose-dependent response of virus inoculation, development of viremia, and subsequent development of antibodies (53); these relationships may be species-specific and different for American robins and northern cardinals. Variability in herd immunity in the adult population could also limit WNv incidence in hatch year individuals (54).

Our analyses highlight the complexities of linking field data of enzootic WNv transmission to the risk of human exposure to WNv. Beyond dead bird surveys (which are currently an uncommon surveillance tool for WNv in most U.S. states) (55-58), few studies have attempted to link prevalence of infections in birds to the risk of WNv exposure in humans (54). Patterns of WNv spillover into human populations are different between Chicago and Atlanta, and in general, spillover of WNv in the southern U.S. is much lower and more sporadic than in

the Midwest (45, 46). However, if intensity of enzootic transmission is indicative of human risk of WNv exposure, our seroincidence estimates would predict far more spillover in Atlanta than in Chicago. Between 2005 and 2012 in Cook County, IL (in which Chicago is located; population approx. 5.2 million), 588 cases of WNv neuro- and nonneuro-invasive disease were reported with neuro-invasive disease case rates varying between 0.41 and 2.31 per 100,000 residents (59). Between 2010 and 2016 in Fulton County, GA (where Atlanta is located; population approximately 1.0 million), only 19 human cases were reported and neuro-invasive disease rates varied between 0.0 and 0.6 per 100,000 residents (59). Other metrics of WNv transmission such as drought and high summer temperatures as well as WNv infection rates in mosquitoes have been linked to human spillover (44, 60, 61). Whether seroincidence rates in hatch year birds are indicative of mosquito WNv infection rates warrants further study. Additionally, other socio-demographic factors such as housing age, income, and time spent indoors at night may better explain the risk of human exposure to WNv in each ragion than estimates of enzootic WNv transmission (62-64).

West Nile virus is an ecologically complex pathogen, and our results provide long-term evidence of variable rates of WNv incidence in host communities. In both Chicago and Atlanta, hatch year birds provide a robust seasonal source of susceptible hosts for infectious, host seeking vectors during WNv epidemic periods. The seasonal breeding dynamics of birds as well as the presence of certain host species are important ecological factors that influence the periodicity of seasonal WNv cycles, yet other extrinsic and ecological factors may mediate the intensity of enzootic transmission within a given season. Additionally, mildly competent species such as northern cardinals seem to play an overall greater role in the transmission dynamics of WNv

across their distribution than predicted through mosquito blood meal and host competency studies.

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Tables and Figures

Table 1. Overall WNv antibody prevalence in hatch year birds from the 10 most commonly sample species in Atlanta, GA and Chicago, IL.

Atlanta (data: 2010 – 2016)				**Chicago (data: 2005 – 2012)**			
Species	N	% Sample	% WNv +	Species	N	% Sample	% WNv +
TOTAL	324	NA	18.2	TOTAL	2,490	NA	7.3
American Robin	92	28.4	12.0	House Sparrow	1110	44.3	6.8
Northern Cardinal	62	19.1	32.3	American Robin	532	21.3	3.9
Northern Mockingbird	42	13.0	40.5	Gray Catbird	145	5.8	12.4
Gray Catbird	24	7.4	25.0	Northern Cardinal	108	4.3	36.1
European Starling	21	6.5	4.8	Song Sparrow	85	3.4	1.2
Brown Thrasher	13	4.0	0.0	House Finch	83	3.3	9.6
Eastern Bluebird	10	3.1	10.0	European Starling	75	3.0	5.3
Carolina Wren	10	3.1	10.0	Swainson's Thrush	72	2.9	1.4
Eastern Towhee	8	2.5	0.0	White-throated Sparrow	34	1.4	0.0
Song Sparrow	7	2.2	0.0	Mourning Dove	22	0.9	27.3

Table 2. Predicted WNv seroincidence curves in hatch year birds in Chicago, IL 2005 – 2012 and Atlanta, GA 2010 – 2016.

Model	***City***	***Intercept[#] (Range)***	***Week Coefficient***	***Odds Ratio (95% CI)***
Independent	Atlanta	-3.2 (-3.97, -2.29)	0.23	1.26 (1.17 – 1.38)
	Chicago	-4.93 (-6.91, -3.03)	0.20	1.23 (1.17 – 1.29)
Data Overlap 2010 - 2012	Atlanta	-3.24 (-4.05, -1.53)	0.31	1.37 (1.23 – 1.54)
	Chicago	-5.00 (-5.81, -3.29)		

[#] Range represents intercept values corrected for Year as a fixed effect; corrected for Year and City for the data overlap analysis.

Figure Legends

Figure 1. Predicted probability any individual hatch year tests positive for WNv by year in Chicago, IL 2005 – 2012. Points represent observed values (0 – Antibody negative, 1 – Antibody Positive) while lines represent the predicted probabilities from a binomial general linear mixed effects model with week and year as fixed effects. Dashed lines represent the lowest and highest 95% confidence limits across all years.

Figure 2. Predicted probability any individual hatch year tests positive for WNv by year in Atlanta, GA 2010 – 2016. Points represent observed values (0 – Antibody negative, 1 – Antibody Positive) while lines represent the predicted probabilities from a binomial general linear mixed effects model with week and year as fixed effects. Dashed lines represent the lowest and highest 95% confidence limits across all years.

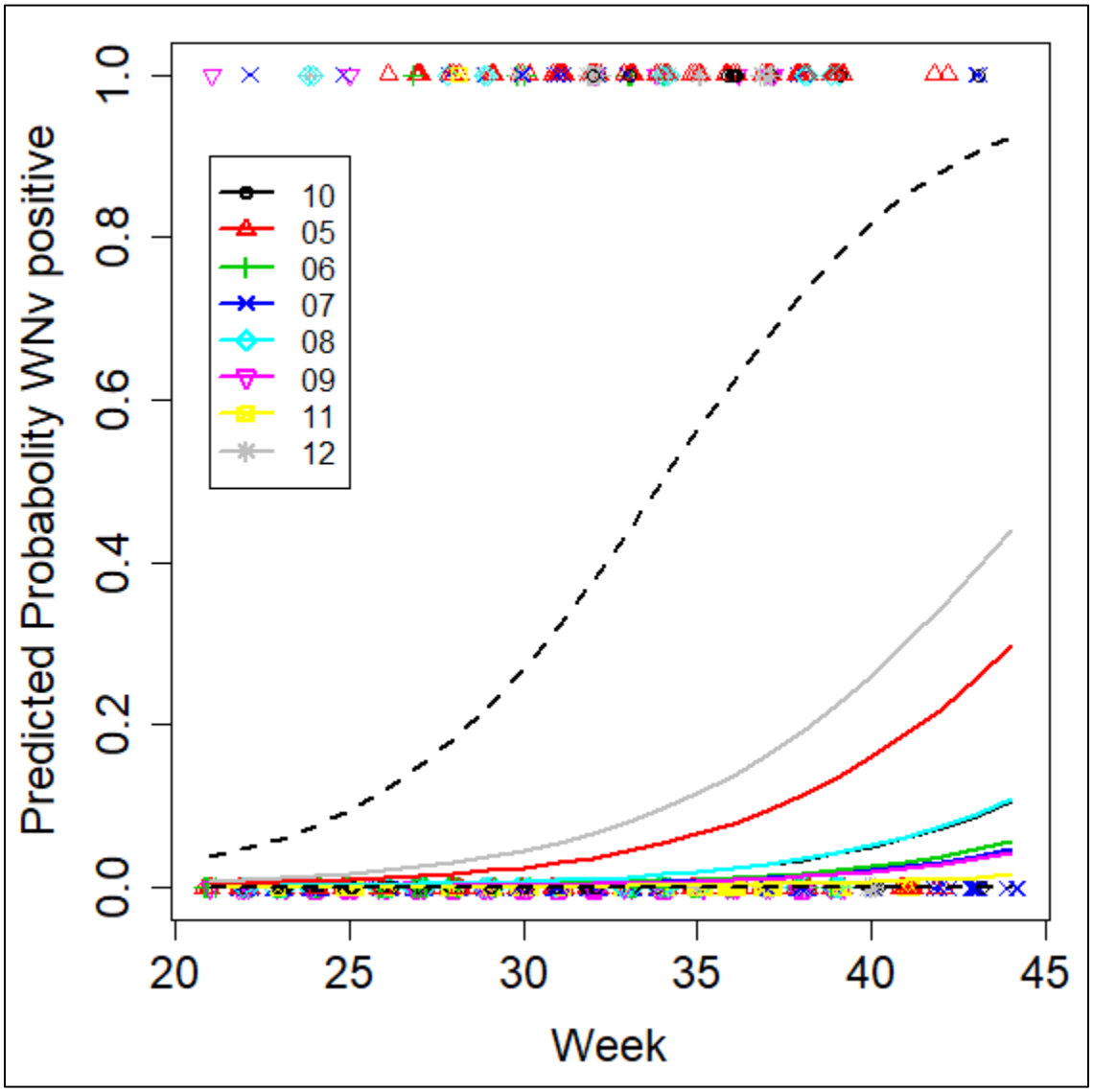
Figure 3. Predicted probability a hatch year bird of a sampled species tests positive for WNv in Chicago, IL 2010. Points represent observed values (0 – Antibody negative, 1 – Antibody Positive) while lines represent the predicted probabilities from a binomial general linear mixed effects model with week as a fixed effect and the intercept corrected for the species-specific random effects terms.

Figure 4. Predicted probability a hatch year bird of a sampled species tests positive for WNv in Atlanta, GA 2010. Points represent observed values (0 – Antibody negative, 1 – Antibody Positive) while lines represent the predicted probabilities from a binomial general linear mixed effects model with week as a fixed effect and the intercept corrected for the species-specific random effects terms.

Figure 5. Predicted probability any individual hatch year tests positive for WNv by year in Chicago, IL and Atlanta, GA 2010 – 2012. Points represent observed values (0 – Antibody negative, 1 – Antibody Positive) while lines represent the predicted probabilities from a binomial general linear mixed effects model with week, city, and year as fixed effects.

Figure 6. Predicted probability a hatch year bird of a sampled species tests positive for WNv in Chicago, IL and Atlanta, GA 2010. Points represent observed values (0 – Antibody negative, 1 – Antibody Positive) while lines represent the predicted probabilities from a binomial general linear mixed effects model with week and city as fixed effects and the intercept corrected for the species-specific random effects terms.

Figure 1.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12

Figure 2.

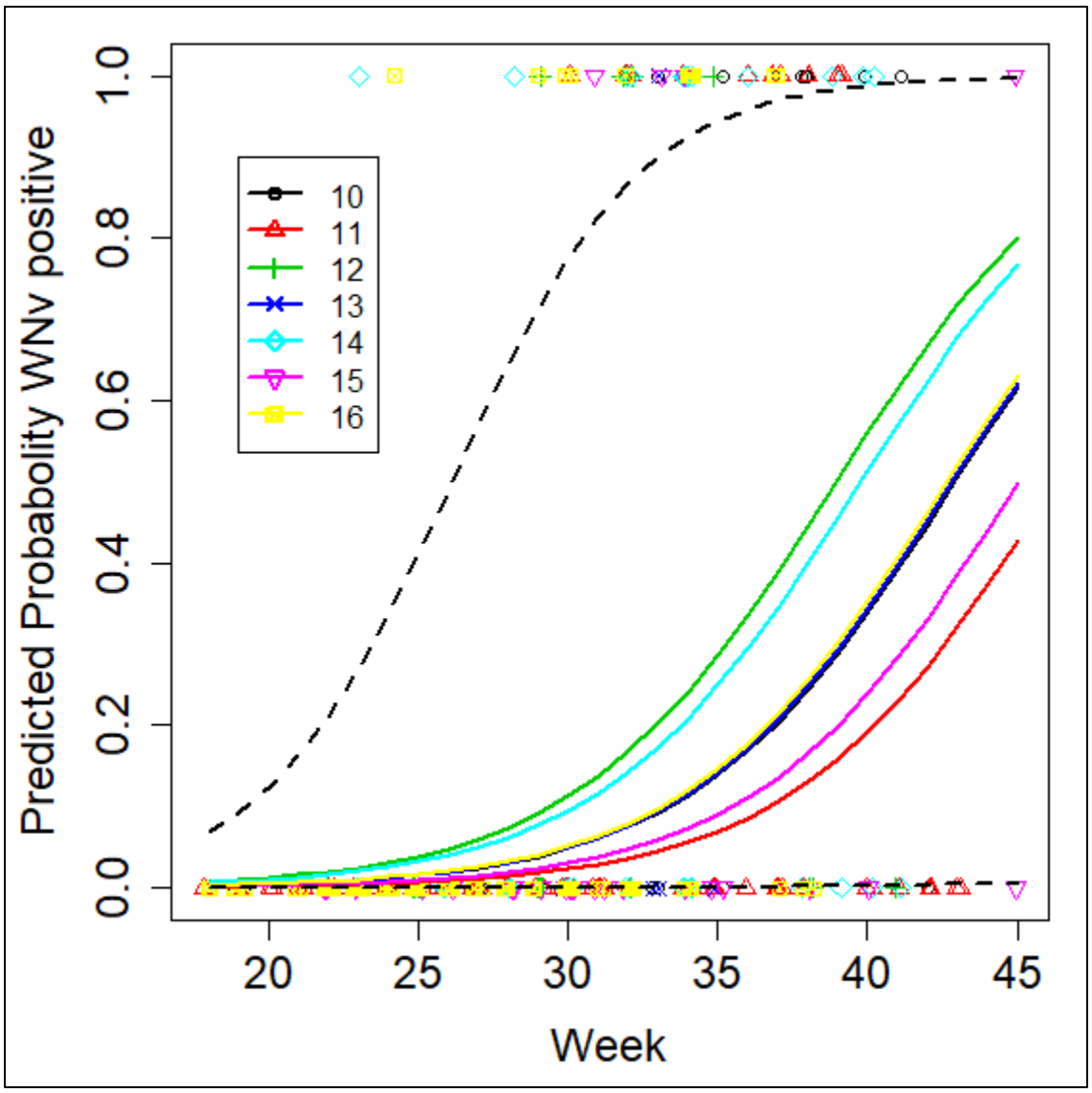
Predicted Probability WNv positive
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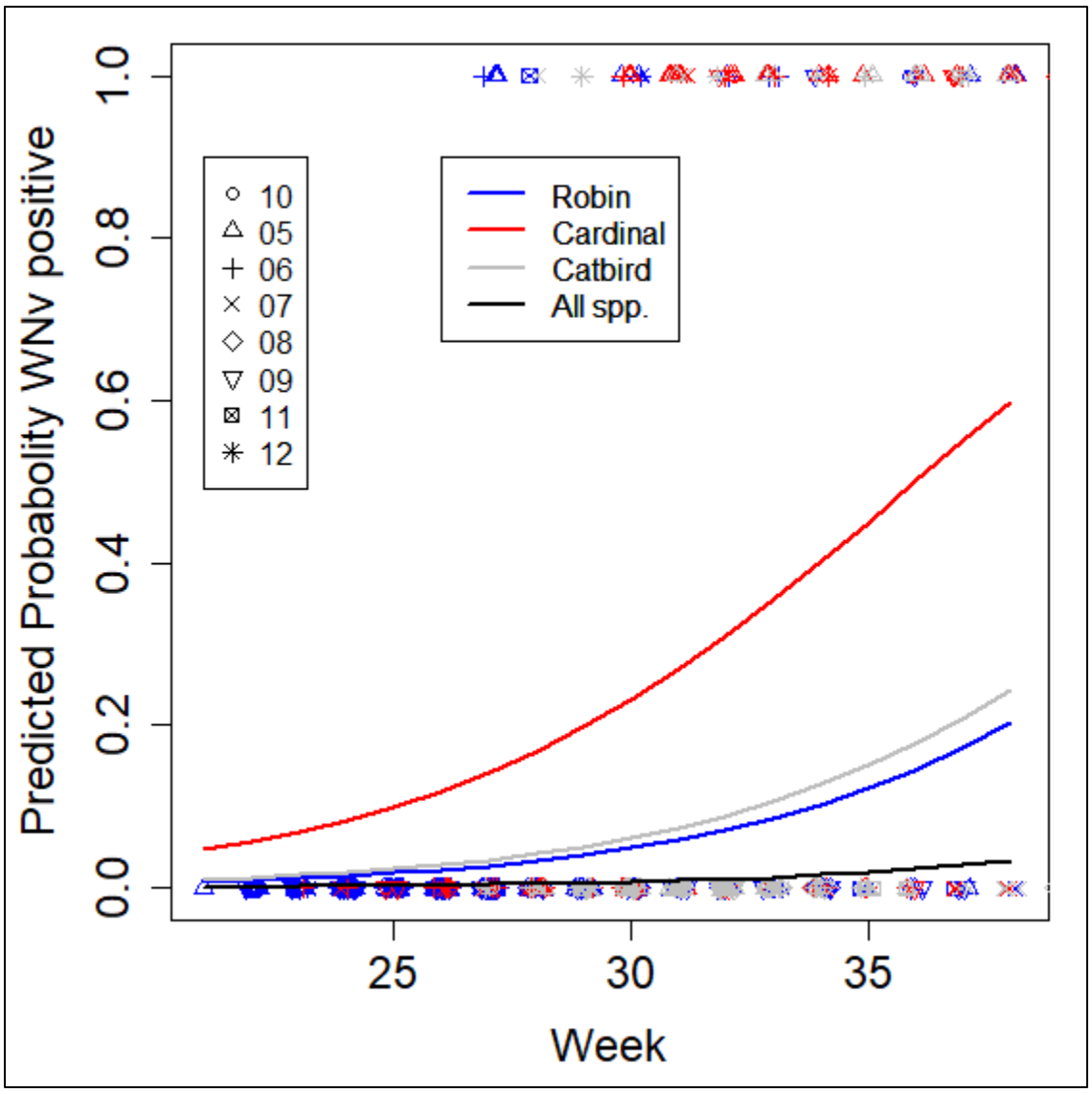
Figure 3.


Predicted Probability WNv positive
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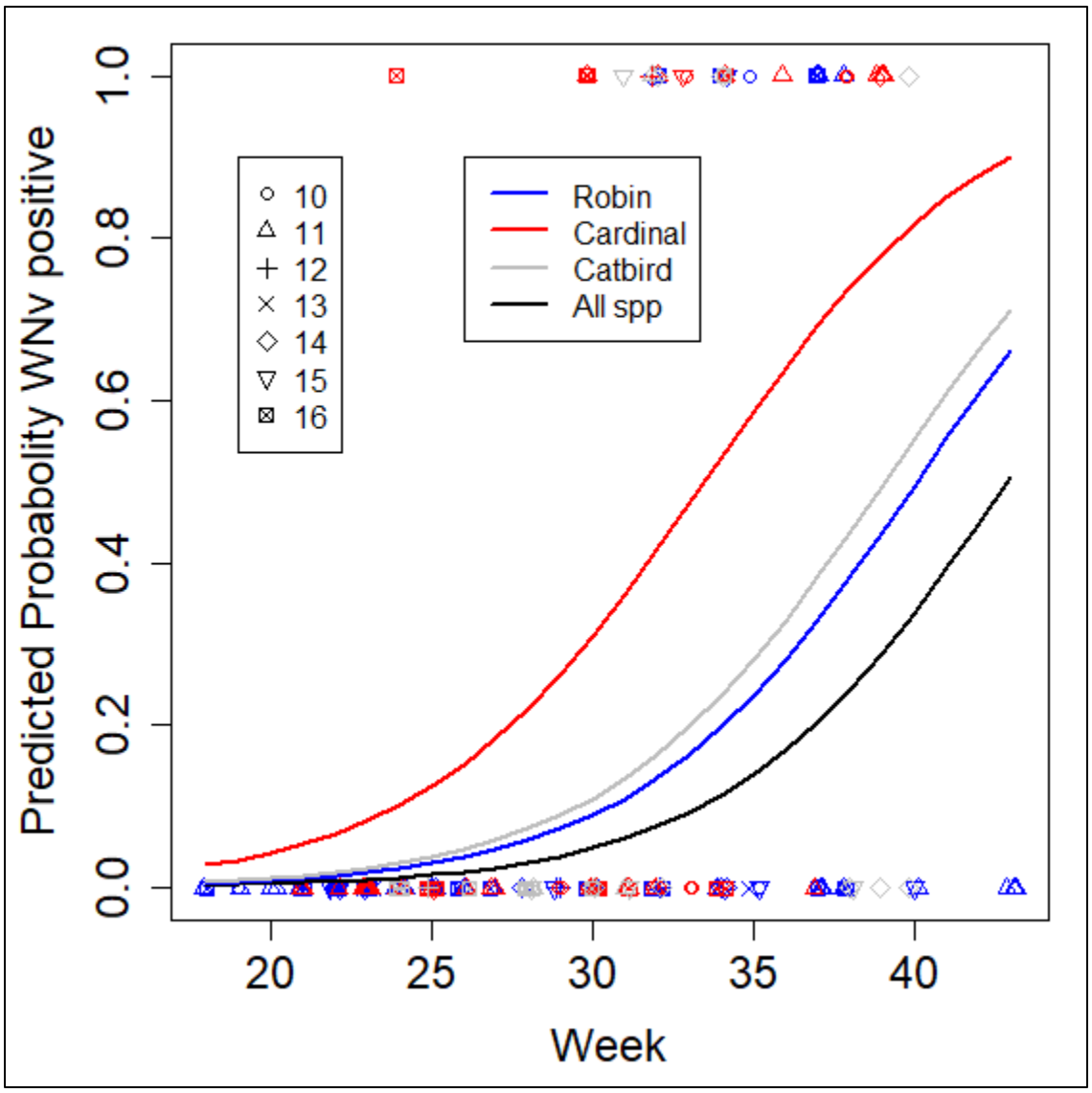
12
Robin
Cardinal
Catbird
All spp.

Figure 4.


Predicted Probability WNv positive
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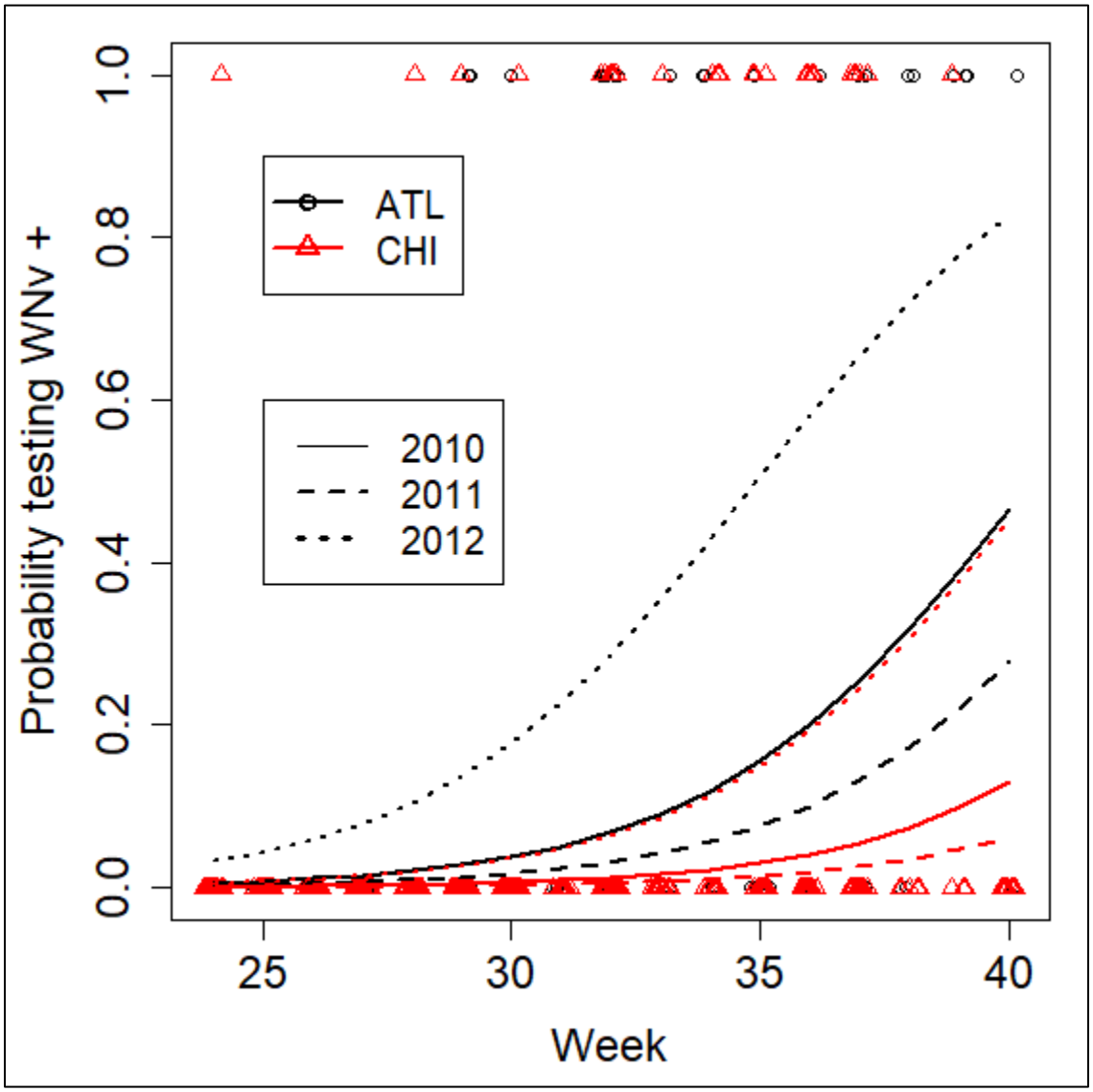
Robin
Cardinal
Catbird
All spp

Figure 5.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Week
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2011
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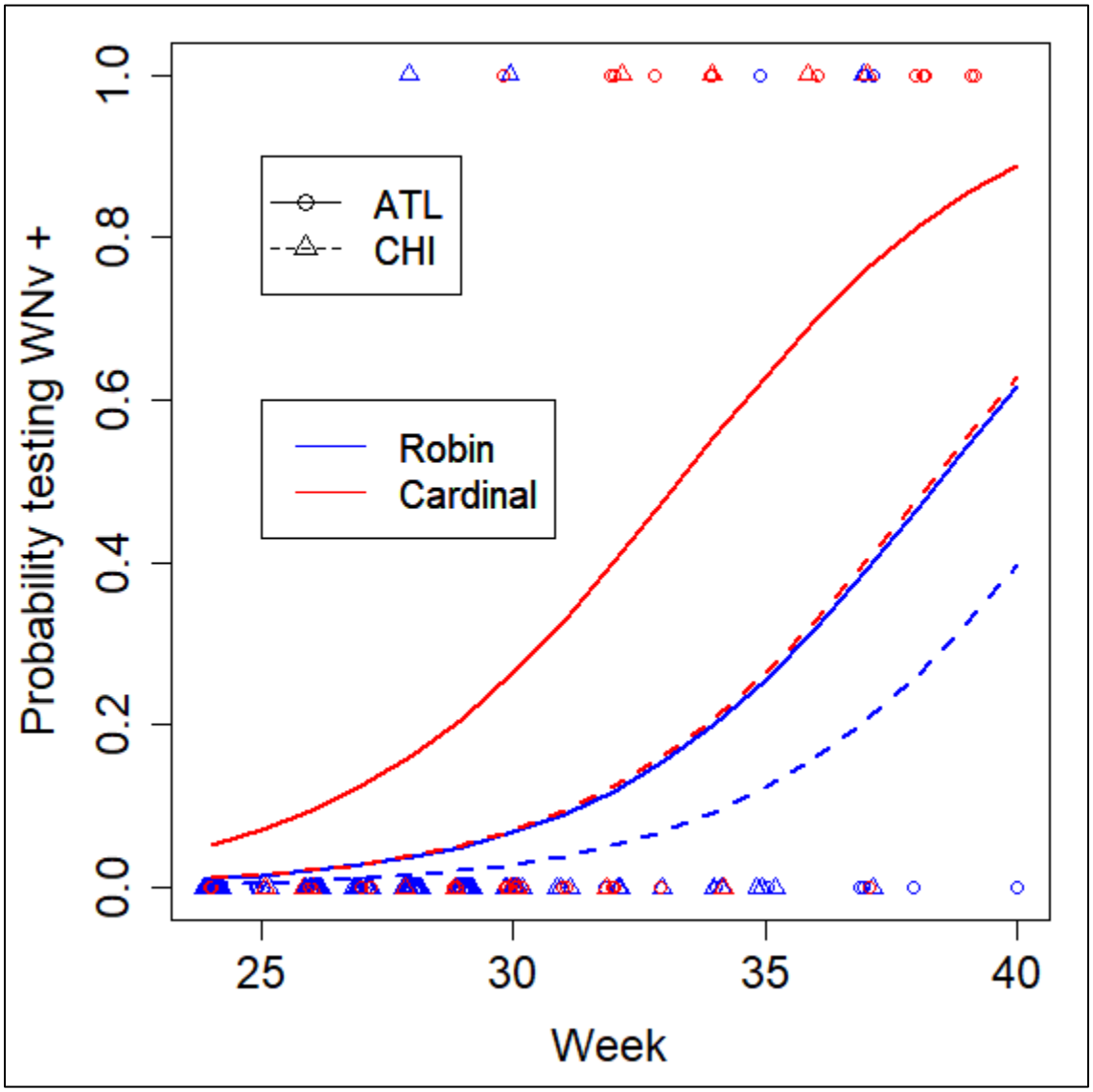
Figure 6.


Probability testing WNv +
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Week
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CHI
Robin
Cardinal

Supplemental Tables and Figure

American Ornithologist's Union (AOU) 4-letter species codes for sampled hatch year species.

Code	Common Name
AMGO	American Goldfinch
AMRE	American Redstart
AMRO	American Robin
AMWO	American Woodcock
BAOR	Baltimore Oriole
BARS	Barn Swallow
BAWW	Black and white Warbler
BBCU	Black-billed Cuckoo
BHCO	Brown-headed Cowbird
BLJA	Blue Jay
BLPW	Blackpoll Warbler
BRTH	Brown Thrasher
CARW	Carolina Wren
CAWA	Canada Warbler
CEDW	Cedar Waxwing
CHSP	Chipping Sparrow
COGR	Common Grackle
COYE	Common Yellowthroat
CSWA	Chestnut-sided Warbler
DOWO	Downy Woodpecker
EABL	Eastern Bluebird

EAPH	Eastern Phoebe
EATO	Eastern Towhee
EAWP	Eastern Woodpeewee
EUST	European Starling
FOSP	Fox sparrow
GCTH	Gray cheeked Thrush
GRCA	Gray Catbird
HAWO	Hairy Woodpecker
HETH	Hermit Thrush
HOFI	House Finch
HOSP	House Sparrow
HOWA	Hooded Warbler
HOWR	House Wren
INBU	Indigo Bunting
LISP	Lincoln's Sparrow
MAWA	Magnolia Warbler
MODO	Mourning Dove
MYWA	Myrtle Warbler
NAWA	Nashville Warbler
NOCA	Northern Cardinal
NOMO	Northern Mockingbird
NOWA	Northern Waterthrush
OVEN	Ovenbird
RBGR	Rose-breasted Grosbeak

RWBL	Red-winged Blackbird
SAVS	Savannah Sparrow
SCJU	Slate-colored Junco
SCTA	Scarlett Tanager
SOSP	Song Sparrow
SWSP	Swamp Sparrow
SWTH	Swainson's Thrush
TRES	Tree Swallow
TUTI	Tufted Titmouse
VEER	Veery
WAVI	Warbling Vireo
WCSP	White-crowned Sparrow
WIFL	Willow Flycatcher
WOTH	Wood Thrush
WPWA	Western Palm Warbler
WTSP	White-throated Sparrow
YSFL	Yellow-shafted Flicker

S. Table 1. West Nile virus seroprevalence in the top 10 sampled species in the Atlanta, GA and Chicago, IL data sets

Atlanta (data: 2010 – 2016)				**Chicago (data: 2005 – 2012)**			
Species	**Sample Size**	**% Sample**	**% WNv +**	**Species**	**Sample Size**	**% Sample**	**% WNv +**
TOTAL	1,085	*NA*	30.0	TOTAL	5,565	*NA*	9.7
American Robin	245	22.3	22.4	House Sparrow	1986	35.7	8.8
Northern Cardinal	204	18.8	53.9	American Robin	1022	18.4	8.4
Northern Mockingbird	97	8.9	46.4	Gray Catbird	384	7.0	11.5
Gray Catbird	91	8.4	37.4	American Goldfinch	259	4.7	5.8
Brown Thrasher	73	6.7	37.4	Northern Cardinal	253	4.5	47.0
Carolina Wren	68	6.3	20.6	Song Sparrow	207	3.7	2.0
Common Grackle	40	3.7	32.5	Red-winged Blackbird	155	2.8	9.0
European Starling	40	3.7	2.5	House Finch	137	2.5	14.6
Blue Jay	23	2.1	47.8	Swainson’s Thrush	132	2.4	0.7
Swainson’s Thrush	23	2.1	4.3	European Starling	131	2.4	7.6

S. Table 2. Results from a binomial-error general linear mixed effects model comparing WNv seroincidence by Week of sampling and Year for all sampled birds in Chicago, IL from 2005 – 2012. The reference year is '2010'.

Fixed Effects

Variable	***Estimate***	***Std. Error***	***Z value***	***Pr (> |z|)***	***Odds Ratio (95% CI)***
Intercept	-4.93	0.70	-7.06	< 0.0001	0.01 (0.001 – 0.02)
Week, centered	0.20	0.02	8.33	< 0.0001	1.22 (1.17 – 1.29)
Year: 2005	1.27	0.35	3.60	0.0003	3.54 (1.84 – 7.38)
Year: 2006	-0.68	0.43	-1.56	0.12	0.51 (0.22 – 1.21)
Year: 2007	-0.88	0.41	-2.11	0.03	0.42 (0.19 – 0.96)
Year: 2008	0.02	0.47	0.04	0.97	1.02 (0.40 – 2.55)
Year: 2009	-0.97	0.59	-1.95	0.05	0.38 (0.14 – 1.00)
Year: 2011	-1.98	1.07	-1.86	0.06	0.14 (0.01 – 0.75)
Year: 2012	1.90	0.43	4.46	< 0.0001	6.67 (2.95 – 15.8)

Random Effects

Groups	***Name***	***Variance***	***Std. Dev.***
Observations (n = 2,490)			
Species (n = 54)	Intercept	4.69	2.17

S. Table 3. Results from a binomial-error general linear mixed effects model comparing WNv sero-incidence by Week of sampling and Year for all sampled birds in Atlanta, GA from 2010 – 2016. The reference year is '2010'.

Fixed Effects

Variable	***Estimate***	***Std. Error***	***Z value***	***Pr (> |z|)***	***Odds Ratio (95% CI)***
Intercept	-3.20	0.89	-3.61	0.0003	0.04 (0.004 – 0.18)
Week, centered	0.23	0.04	5.61	< 0.0001	1.26 (1.17 – 1.38)
Year: 2011	-0.77	0.63	-1.23	0.22	0.46 (0.14 – 1.62)
Year: 2012	0.91	0.84	1.09	0.28	2.49 (0.49 – 13.6)
Year: 2013	0.02	1.05	0.02	0.98	1.02 (0.11 – 7.5)
Year: 2014	0.72	0.75	0.96	0.34	2.06 (0.48 – 9.19)
Year: 2015	-0.49	0.87	-0.57	0.57	0.61 (0.10 – 3.25)
Year: 2016	0.06	0.65	0.09	0.93	1.06 (0.30 – 3.94)

Random Effects

Groups	***Name***	***Variance***	***Std. Dev.***
Observations (n = 324)			
Species (n = 26)	Intercept	1.55	1.25

S. Table 4. Results from binomial-error general linear mixed effects model comparing WNv seroincidence across all sampled species by Week of sampling, Year, and City for 2010 – 2012. The reference Year is 2010 and the reference city is 'Atlanta'.

Fixed Effects

Variable	***Estimate***	***Std. Error***	***Z value***	***Pr (> |z|)***	***Odds Ratio (95% CI)***
Intercept	-3.24	0.92	-3.54	0.0004	0.04 (0.003 – 0.17)
Week, centered	0.31	0.06	5.48	< 0.0001	1.37 (1.23 – 1.54)
City: Chicago	-1.76	0.84	-2.10	0.04	0.17 (0.03 – 0.99)
Year: 2011	-0.81	0.49	-1.65	0.10	0.44 (0.16 – 1.14)
Year: 2012	1.71	0.42	4.06	< 0.0001	5.55 (2.48 – 13.1)

Random Effects

Groups	***Name***	***Variance***	***Std. Dev.***
Observations (n = 528)			
Species by City (n = 57)	Intercept	2.94	1.71
City (n = 2)	Intercept	0.000	0.000

Figure Legends

S. Figure 1. Sample sites in A) Chicago, IL and B) Atlanta, GA. Scale bar is for both county maps. See Hamer et al. 2008 and Levine et al. 2016 for specific sampling locations within the sampling boundary.

S. Figure 2. Proportion of hatch year individuals identified as American robin (AMRO), northern cardinal (NOCA), or house sparrow (HOSP) in Chicago, IL 2005 - 2012. Points represent observed values while thick lines represent the average value across years.

S. Figure 3. Proportion of hatch year individuals identified as American robin (AMRO), northern cardinal (NOCA), or northern mockingbird (NOMO) in Atlanta, GA 2010 – 2016. Points represent observed values while thick lines represent the average value across years.

S. Figure 4. Marginal effects for fixed effect term 'cWeek' from a binomial general linear mixed effects model for WNv seroincidence in hatch year birds from Chicago, IL 2005 - 2012. 'cWeek' – week variable centered to July

S. Figure 5. Marginal effects for fixed effect term 'cWeek' from a binomial general linear mixed effects model for WNv seroincidence in hatch year birds from Atlanta, GA 2010 - 2016. 'cWeek' – week variable centered to July

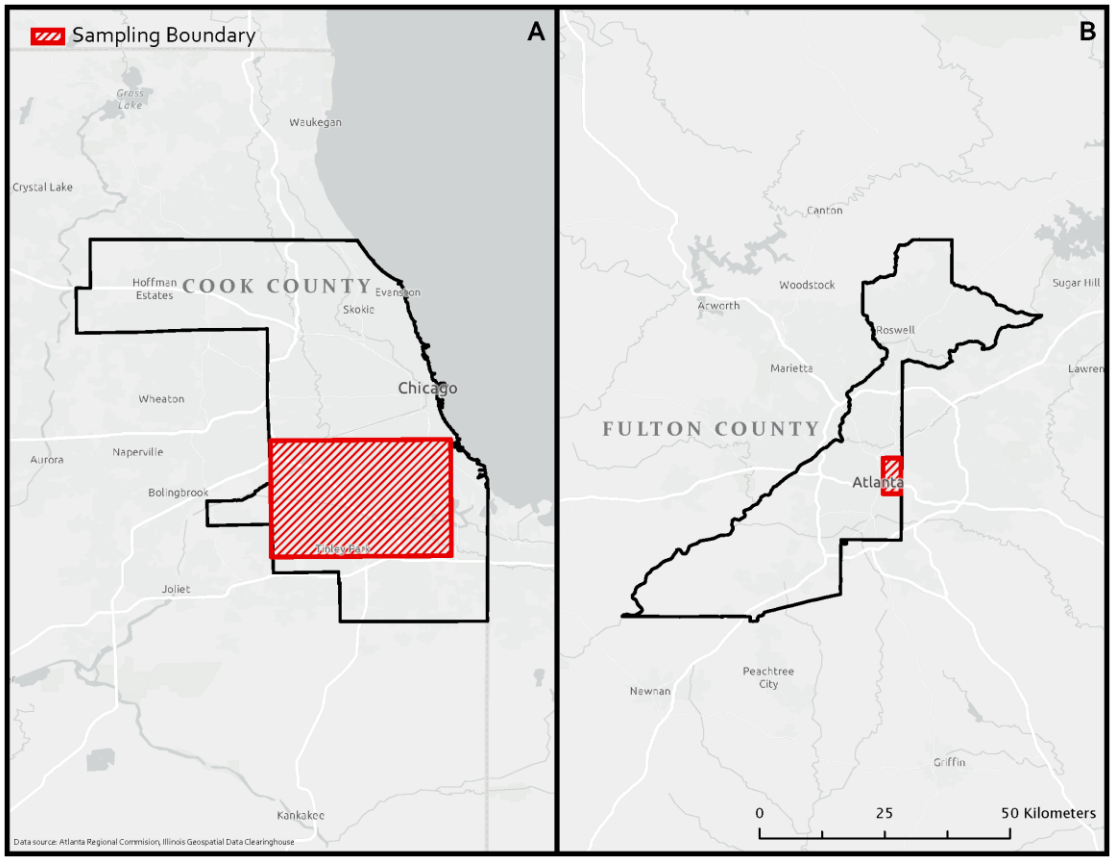
S. Figure 6. Odds ratios for random effects from a binomial general linear mixed effects model for WNv seroincidence in hatch year birds from Chicago, IL, 2005 – 2012. See Species List for abbreviation names

S. Figure 7. Odds ratios for random effects from a binomial general linear mixed effects model for WNv seroincidence in hatch year birds from Atlanta, GA 2010-2016. See Species List for abbreviation names

S. Figure 8. Marginal effects for fixed effect 'cWeek' and City from a binomial general linear mixed effects model for WNv seroincidence in hatch year birds from Chicago, IL and Atlanta, GA 2010 – 2012. 'cWeek' – week variable centered to July.

S. Figure 9 Odds ratios for random effects from a binomial general linear mixed effects model for WNv seroincidence in hatch year birds from Chicago, IL and Atlanta, GA 2010 – 2012. See Species lists for abbreviation names

S. Figure 1.


Sampling Boundary
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COOK COUNTY
Evanston
Skokie
Chicago
Wheaton
Naperville
Aurora
Bolingbrook
Joliet
Kankakee
Data source: Atlanta Regional Commision, Illinois Geospatial Data Clearinghouse
Canton
Woodstock
Sugar Hill
Acworth
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FULTON COUNTY
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Newnan
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0
25
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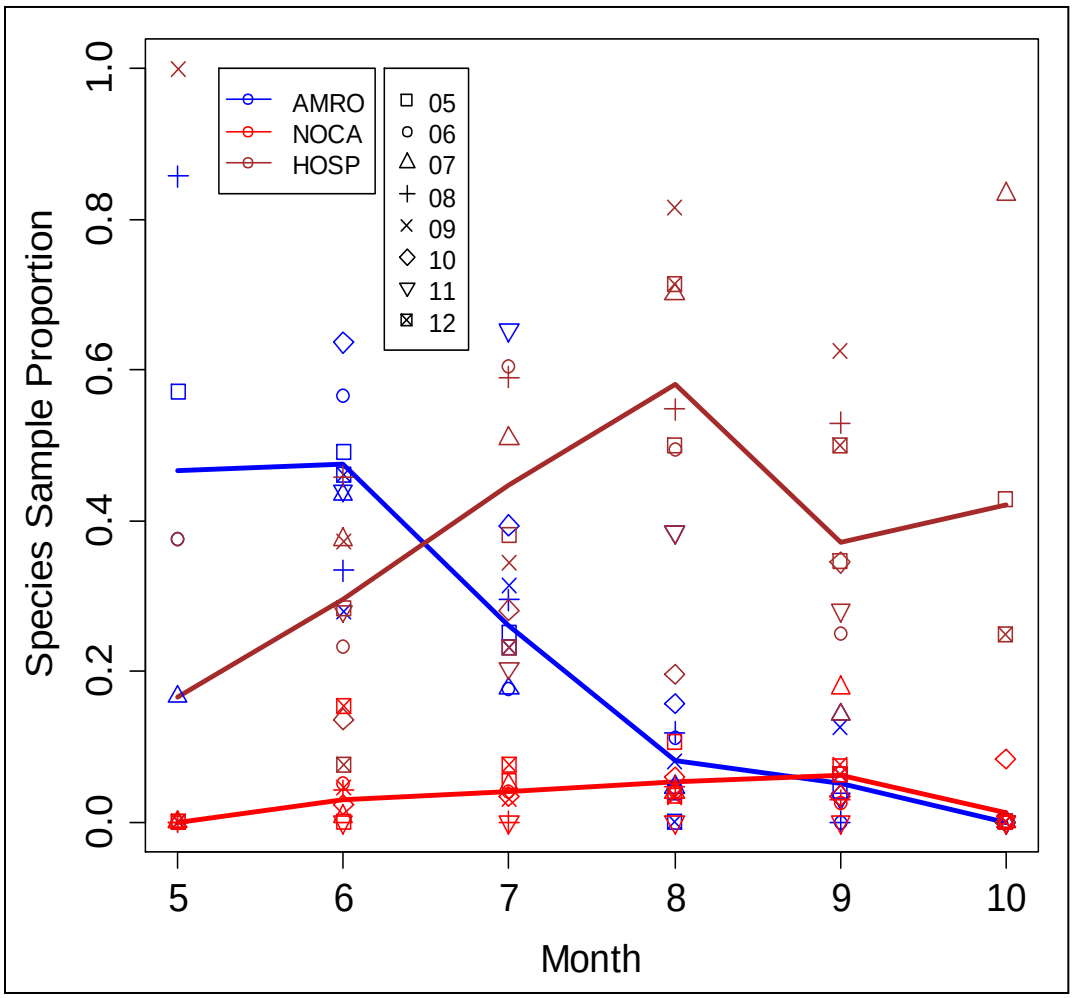
S. Figure 2.


AMRO
NOCA
HOSP
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Species Sample Proportion
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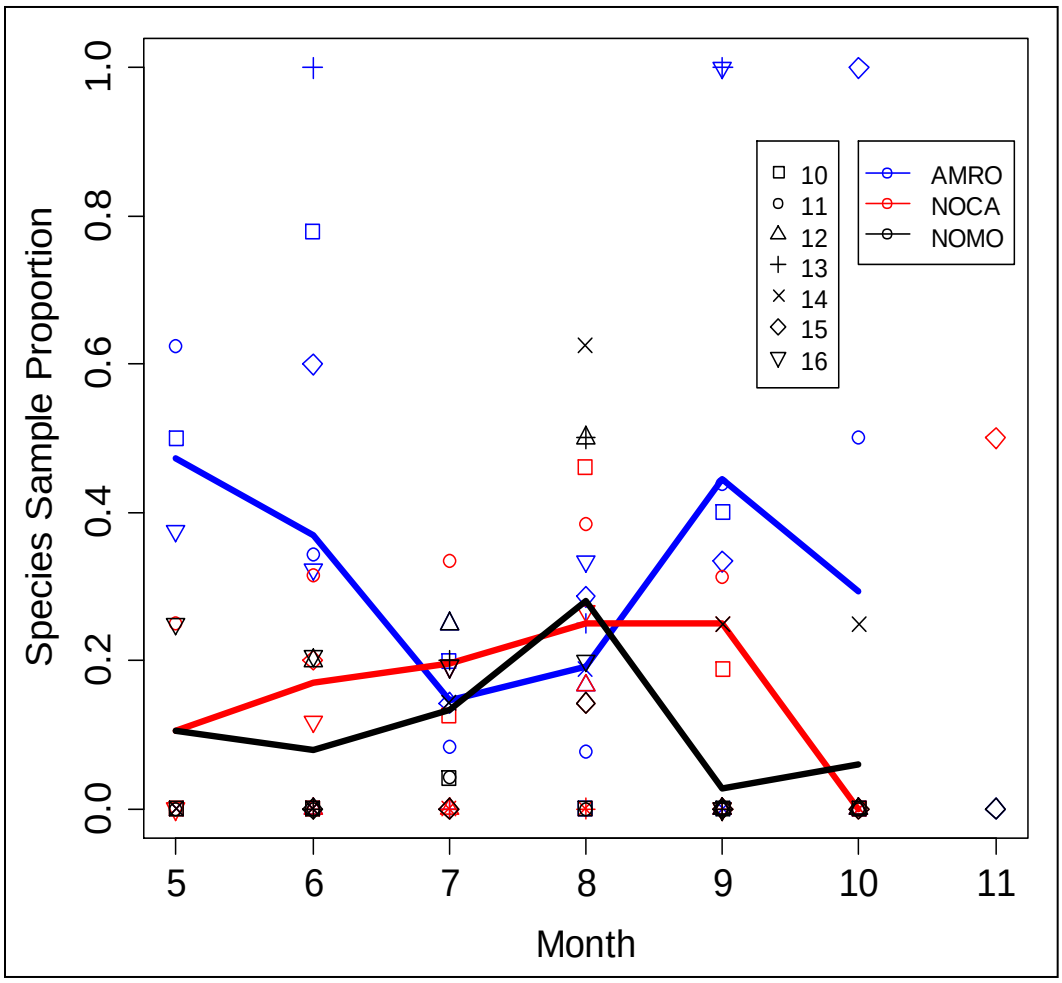
S. Figure 3.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AMRO
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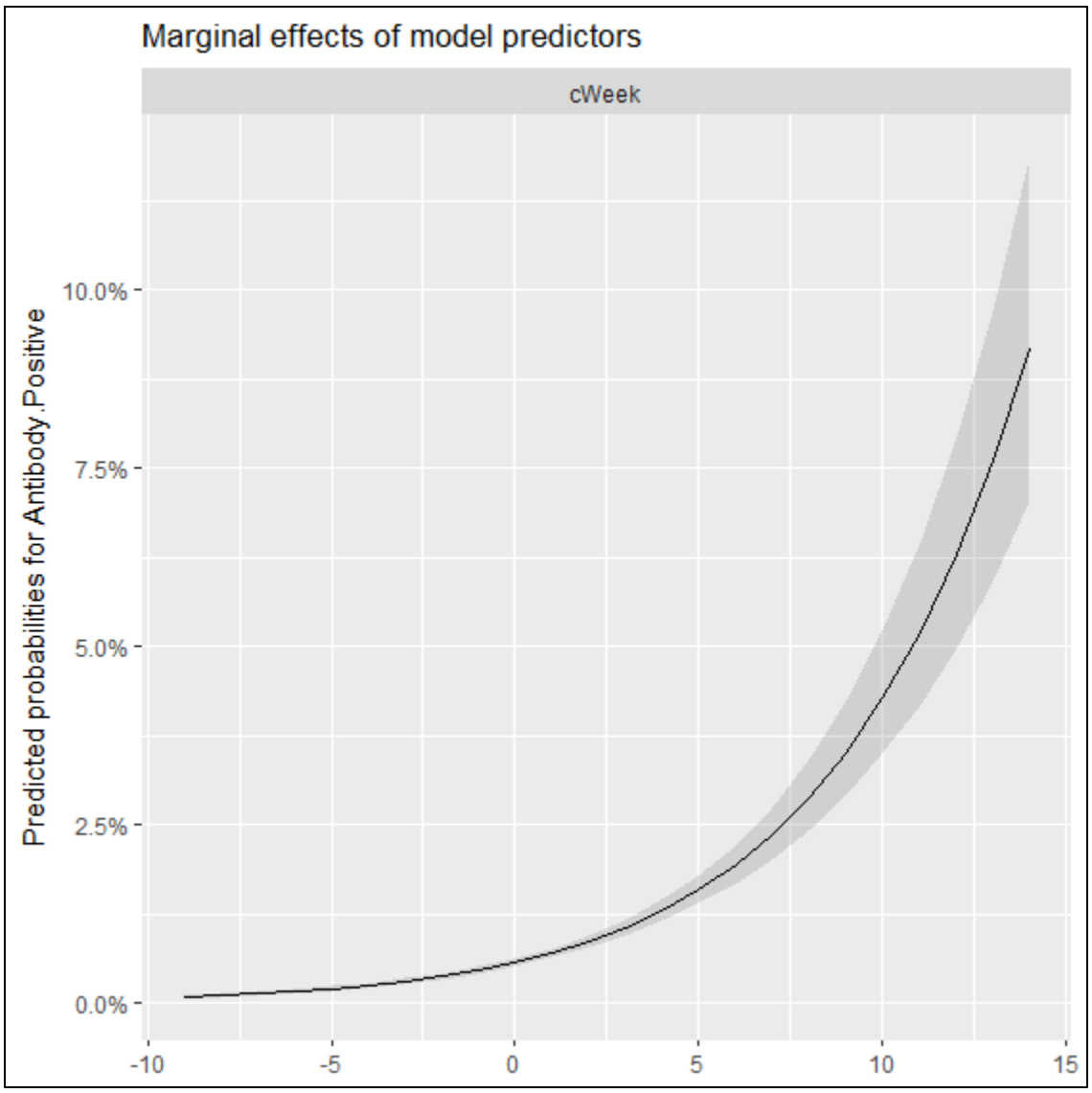
NOMO

S. Figure 4.


Marginal effects of model predictors
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Predicted probabilities for Antibody.Positive
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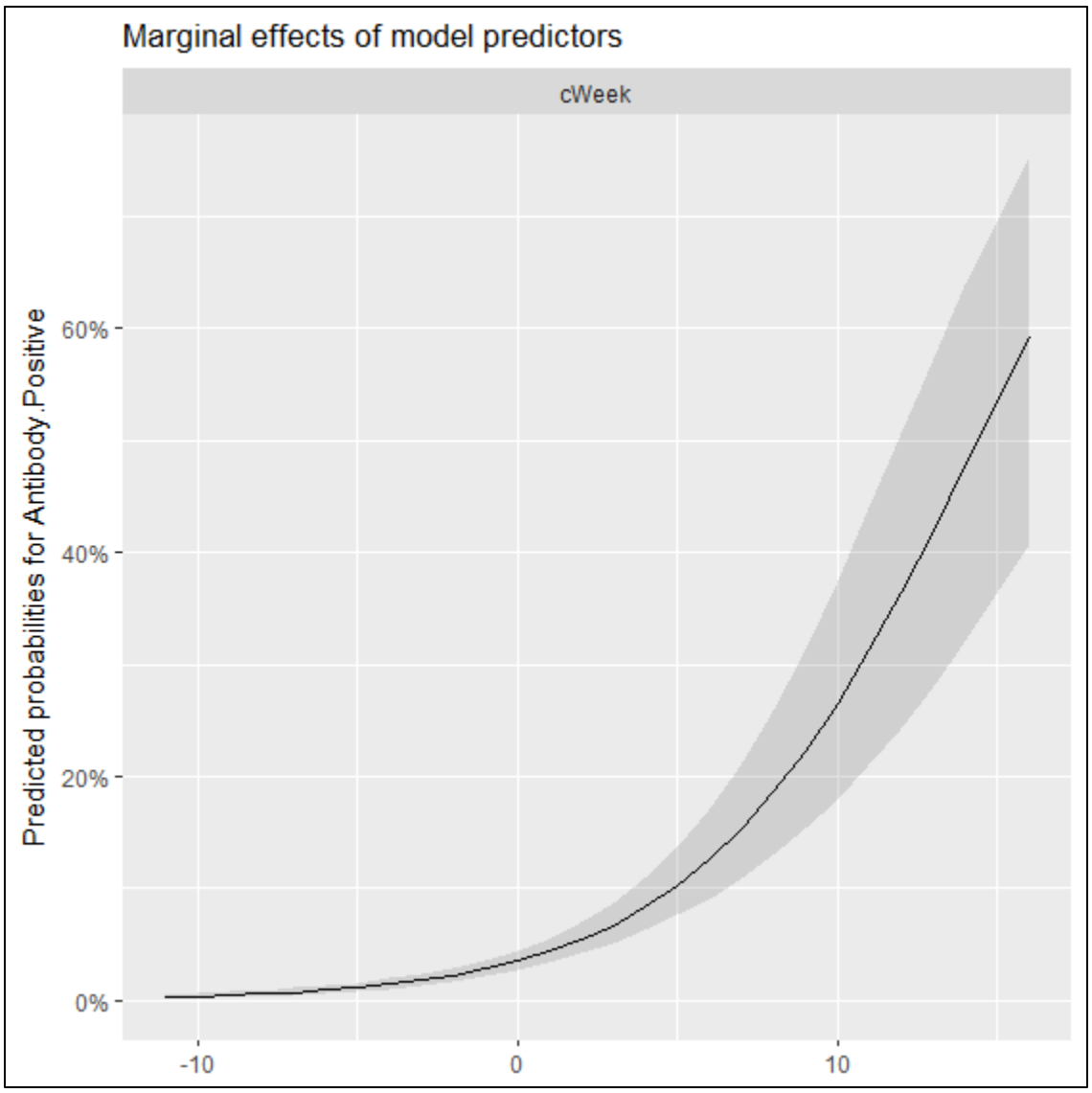
15

S. Figure 5.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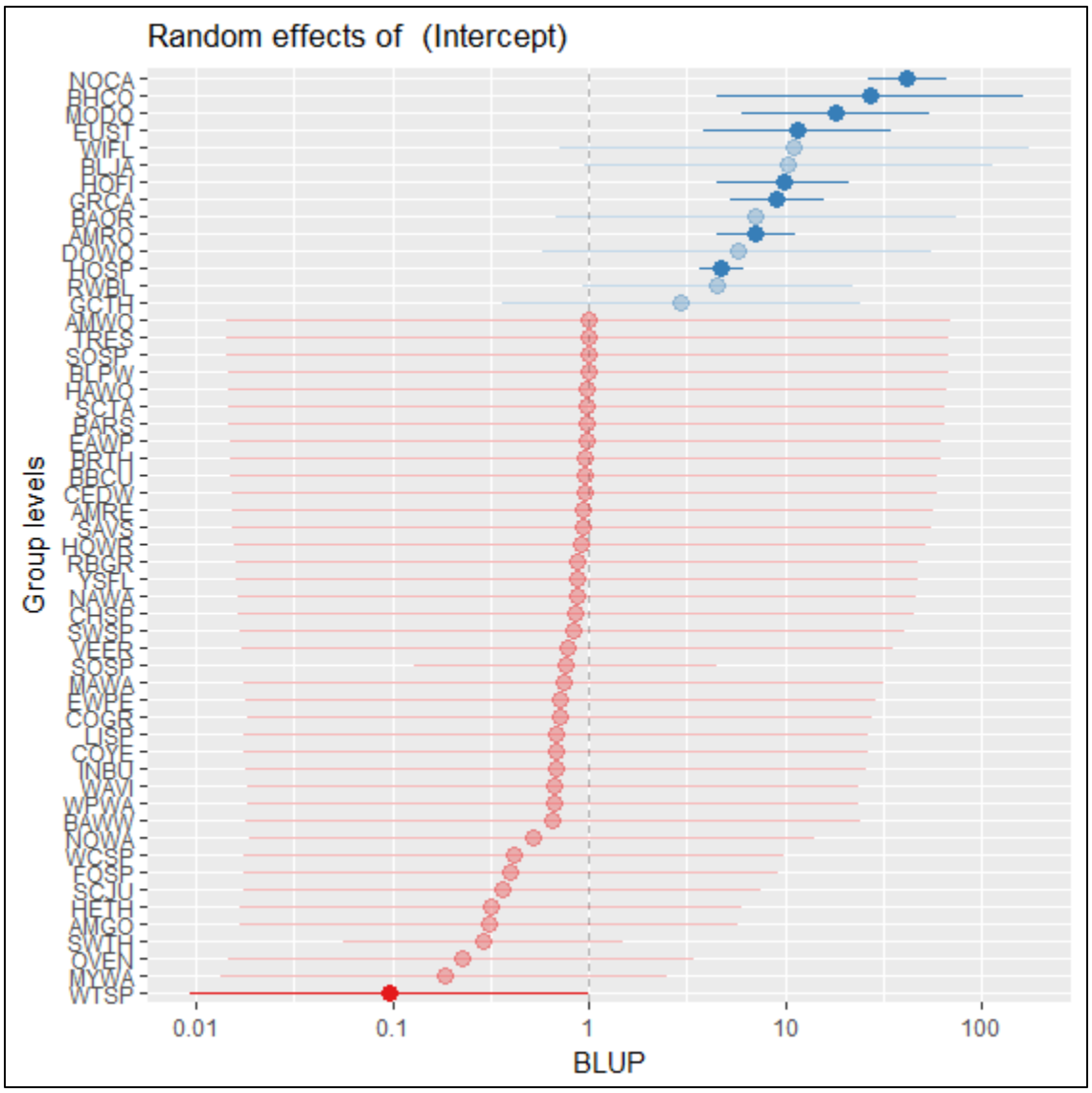
S. Figure 6.


Random effects of (Intercept)
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RWBL
GCTH
AMWO
TRES
SOSP
BLPW
HAWO
SCTA
BARS
EAWP
BRTH
BBCU
CEDW
AMRE
SAVS
HOWR
RBGR
YSFL
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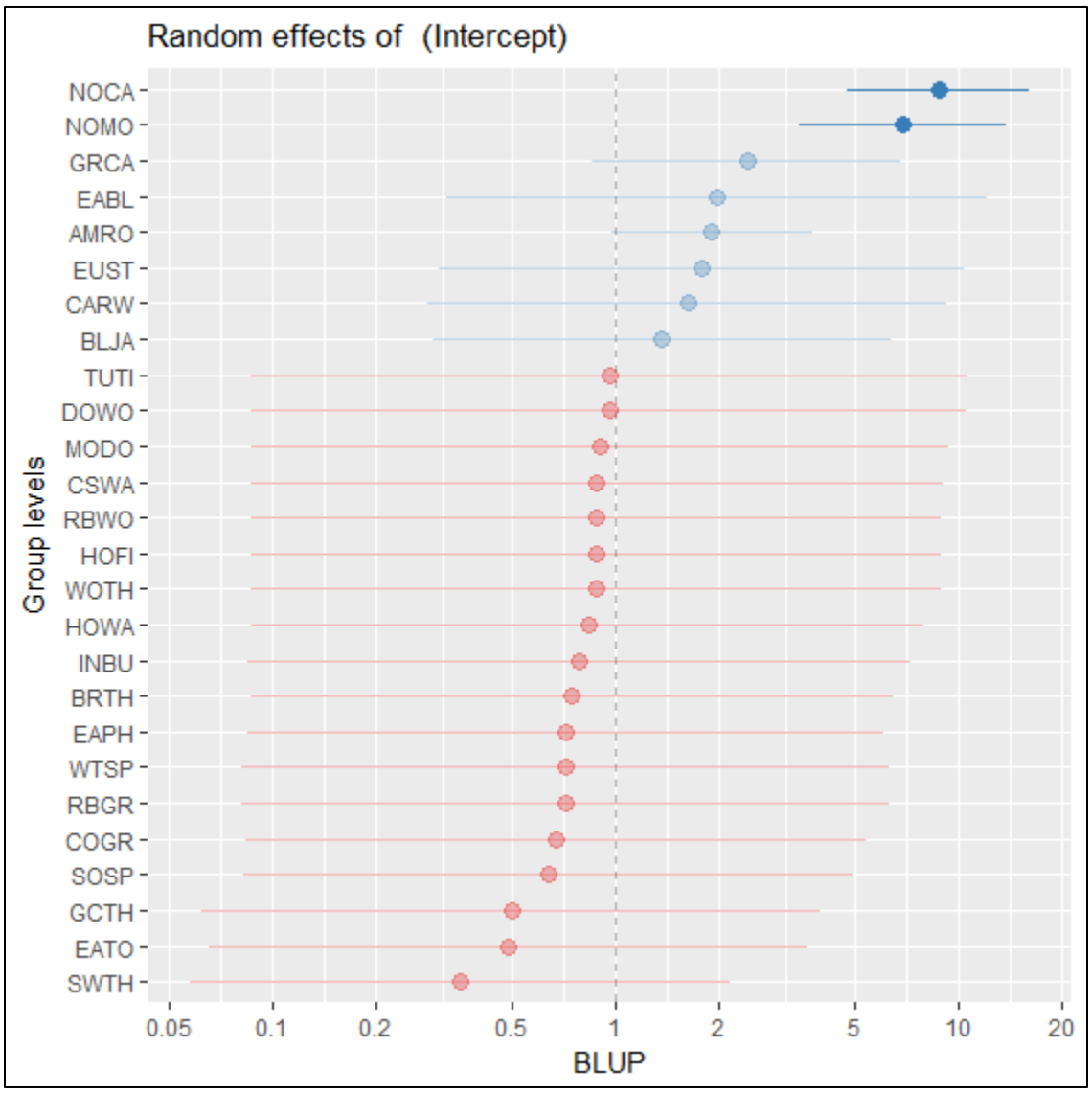
S. Figure 7.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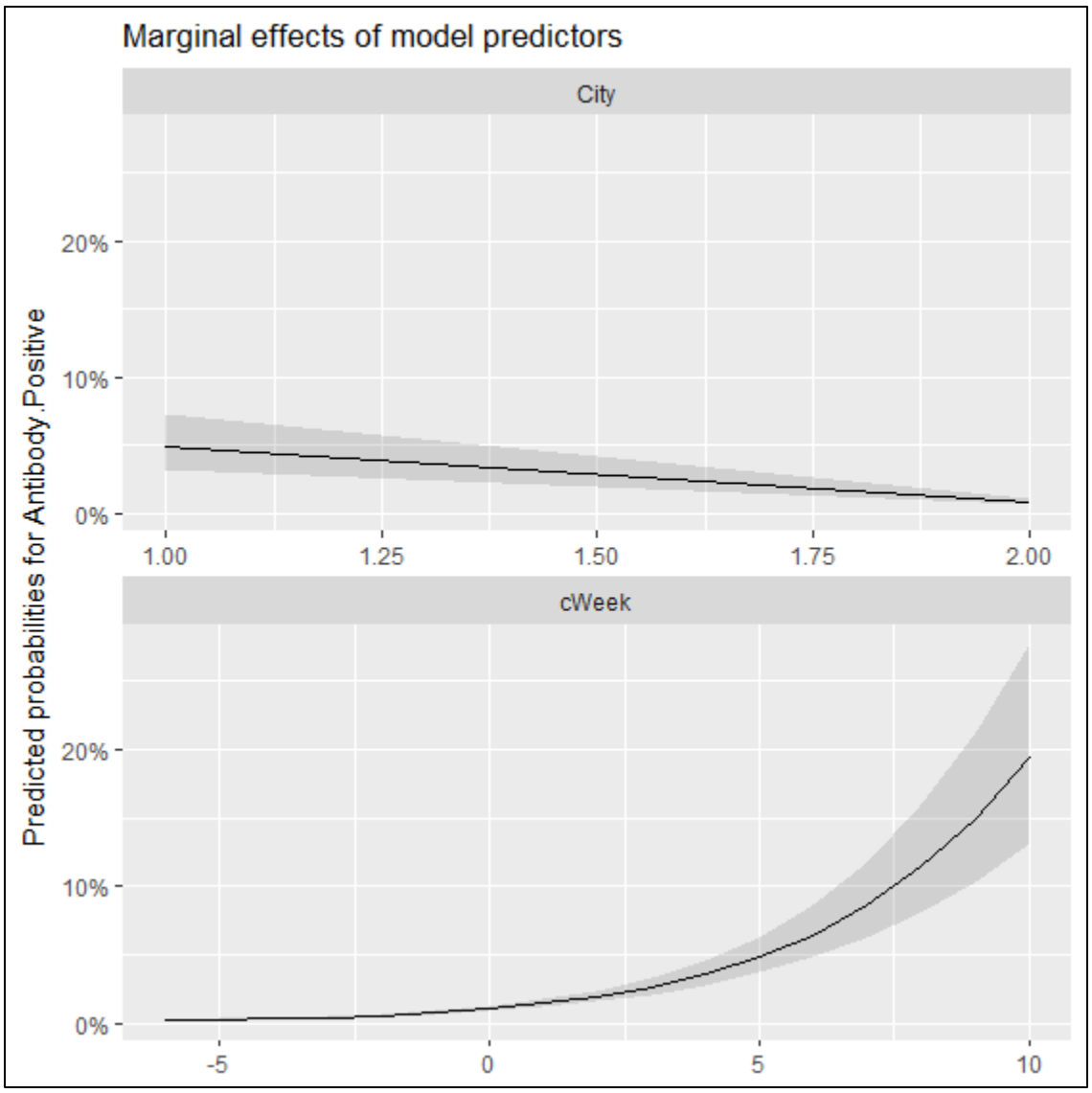
RBWO
HOFI
WOTH
HOWA
INBU
BRTH
EAPH
WTSP
RBGR
COGR
SOSP
GCTH
EATO
SWTH
0.05
0.1
0.2
0.5
1
2
5
10
20
BLUP

S. Figure 8.


Marginal effects of model predictors
City
cWeek
Predicted probabilities for Antibody.Positive
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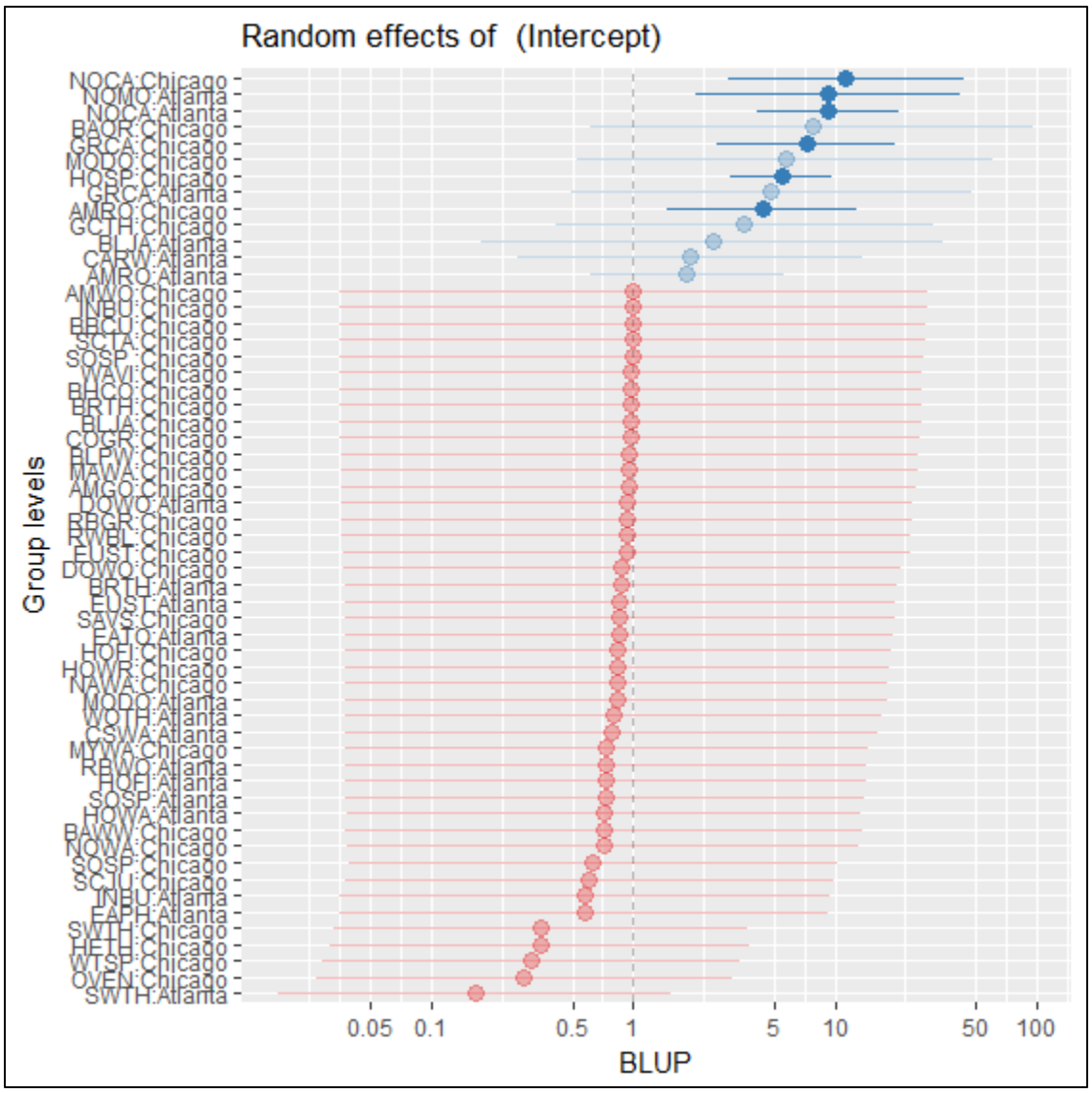
10%
0%
1.00
1.25
1.50
1.75
2.00
-5
0
5
10

S. Figure 9.


Random effects of (Intercept)
Group levels
NOCA:Chicago
NOMO:Atlanta
NOCA:Atlanta
BAOR:Chicago
GRCA:Chicago
MODO:Chicago
HOSP:Chicago
GRCA:Atlanta
AMRO:Chicago
GCTH:Chicago
BLJA:Atlanta
CARW:Atlanta
AMRO:Atlanta
AMWO:Chicago
INBU:Chicago
BRCU:Chicago
SCTA:Chicago
SOSP:Chicago
WAVI:Chicago
BHCO:Chicago
BRTH:Chicago
BLJA:Chicago
COGR:Chicago
BLPW:Chicago
MAWA:Chicago
AMGO:Chicago
DOWO:Atlanta
RBGR:Chicago
RWBL:Chicago
EUST:Chicago
DOWO:Chicago
BRTH:Atlanta
EUST:Atlanta
SAVS:Chicago
EATO:Atlanta
HOFI:Chicago
HOWR:Chicago
NAWA:Chicago
MODO:Atlanta
WOTH:Atlanta
CSWA:Atlanta
MYWA:Chicago
RBWO:Atlanta
HOFI:Atlanta
SOSP:Atlanta
HOWA:Atlanta
BAWW:Chicago
NOWA:Chicago
SOSP:Chicago
SCJU:Chicago
INBU:Atlanta
EAPH:Atlanta
SWTH:Chicago
HETH:Chicago
WTSP:Chicago
OVEN:Chicago
SWTH:Atlanta
0.05
0.1
0.5
1
5
10
50
100
BLUP

Chapter 3: Host Selection and Feeding Success of *Culex quinquefasciatus* mosquitoes in Experimental Trials

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Introduction

Vector-borne zoonotic disease systems are dominated by complex interactions between communities of vectors, pathogens, wildlife reservoirs, and human populations (1). Within these zoonotic communities, successful pathogen transmission is strongly dependent on the detection by the vector of a suitable and competent host, and by the successful acquisition of a blood meal from the host by the vector. In the case of mosquito-borne pathogens, heterogeneities in mosquito-host contacts, referred to as non-random biting or mosquito host preferences, significantly modulate the rate of transmission and amplification of vector-borne diseases (2, 3). Incorporating feeding preferences into the vectorial capacity equation, which estimates the number of infected vectors from a population feeding on a single infected host, reveals that aggregated feeding by vectors on competent hosts greatly increases the probability of successful pathogen transmission (4, 5). Therefore, it is critical to quantify the feeding behaviors of vectors in order to predict the risk of pathogen spread and inform public health and vector control policies.

A common method of estimating mosquito host preferences is through the analysis of field-collected, blood fed adult mosquitoes (6, 7). This method presents many challenges for parameter estimation due to the difficulty of linking a mosquito's host choice with estimates of host availability at the time and place when feeding occurs. Suitable hosts are heterogeneously available in space and time (8) and mosquito host selection patterns occur across a wide range of

spatial scales (9). Hosts also differ in their respective defensive strategies against mosquito biting (10), and previous studies have shown a wide variation in both intra and inter-specific host defensive behaviors (11, 12). Baited traps that quantify the attraction of vectors to different hosts can address issues of host abundance and presence (13, 14). However, these methods are limited in their ability to quantify vector abundance and, for human pathogens such as malaria, place baited individuals at risk of exposure to infected vectors. Experimental host choice studies can complement field studies because they control for host availability, vector: host ratios, and climatic conditions. Despite their utility for quantifying host selection patterns, few studies have utilized controlled experimental methods to quantify mosquito host preferences of different epidemiologically important host species.

In the United States there has been renewed interest in understanding mosquito blood feeding behaviors due to the introduction and rapid spread of West Nile virus (WNv). West Nile virus was first detected in the United States in 1999 in New York City, New York, from where it rapidly spread throughout North America (15). *Culex* spp. mosquitoes (primarily *Culex pipien pipiens* in northern latitudes, *Culex quinquefasciatus* in southern latitudes, and *Culex tarsalis* in Western and Central US) are considered the main vectors, and birds of the order Passeriformes the main reservoirs of the virus (15). In the Eastern and Midwestern US, field studies have shown American robins (*Turdus migratorious*) are an over-utilized and possibly preferred blood meal source for *Cx. pipiens pipiens* mosquitoes (16). These field studies are supported by a host choice experiment in which *Cx. pipiens pipiens* were captured more frequently in host-funnel traps baited with American robins as opposed to European starlings (*Sturnus vulgaris*) and house sparrows (*Passer domesticus*) (17).

Blood feeding behaviors for *Cx. quinquefasciatus* are considered more opportunistic than its sister species, *Cx. pipiens pipiens.* In the Southeastern U.S., *Cx. quinquefasciatus* mosquitoes feed on a wide variety of peri-domestic avian species such as northern mockingbirds (*Mimus polyglottus*), northern cardinals (*Cardinalis cardinalis*), American robins, common grackles (*Quiscalus quiscula*), and gray catbirds (*Dumetella carolinensis*) (18-20). In Atlanta, GA shifts in host selection between American Robins and Northern Cardinals have been proposed as a mechanism explaining infrequent human spillover of WNv (20). It is unknown whether this shift in host choice by *Cx. quinquefasciatus* mosquitoes is due to inherent feeding preferences of the vector, shifts in host species availability, or some other mechanism.

We designed a semi-natural blood feeding experiment to quantify *Cx. quinquefasciatus* host preferences for American Robins and northern cardinals as well as blue jays (*Cyanocitta cristata*), brown thrashers (*Toxostoma rufum*), and gray catbirds. All five species are common peri-urban species, and all five have been characterized as important blood meal hosts in *Cx. quinquefasciatus* mosquitoes in the area (20). Our null hypothesis was that *Cx. quinquefasciatus* blood meal choices would correlate with the availability of hosts, and there would be no detectable preference for any particular species. We then utilized our host preference results to estimate WNv transmission potential in these two-host communities using the vectorial capacity framework taking into account observed *Cx. quinquefasciatus* feeding preferences.

Material and Methods

All field and experimental procedures were approved by Emory University Institute for Animal Care and Use (IACUC DAR-2002351), having been granted local and federal bird collection permits (GA DNR 29-WJH-14-90 and USGS 23673).

Study Design

An aviary was constructed with ½" PVC piping measuring 1.75m x .75m x 0.75m and mosquito netting glued to the pipes (**Figure 1**). A cloth stockinette was attached to one side of the cage, so that a mosquito proof seal could be maintained when adding/removing birds and mosquitoes. One side of the aviary's netting was held to the structure with metal fasteners so that the aviary could be opened to add/remove bird cages. The aviary was placed length-wise on top of a plastic folding table and kept underneath an outdoor pavilion for all experiments. The interior of the aviary allowed two 0.33m x 0.28m x 0.41m metal bird cages (Prevue Pet Products) with 0.013m wire spacing to be inserted within so that birds remained confined to a certain space but still had room to actively defend themselves from mosquito attacks. Mosquitoes had equal access to both birds within the aviary.

Culex quinquefasciatus mosquitoes were reared from field collected egg rafts obtained in Atlanta, Georgia between July and September 2010, 2011, and 2013 using gravid trap bins baited with a mixture of water, hay, and dog food (21). Larvae emerging from eggs were reared at 30°C and fed daily 10g of yeast (Fleishmann's, ACH Food Companies, INC., Memphis, TN) or 20g of crushed Koi food pellets (Tetra Pond, Tetra Holding, INC., Blacksburg, VA). Pupae were removed to an emergence chamber and both male and female adults were provided a 10% sugar/water solution. Female mosquitoes used in the host choice assays were at least 3 days post-emergence and were deprived of the sugar solution approximately 8 hours before each experiment.

Each experiment consisted of pairing a northern cardinal with a bird of another species: an American robin, blue jay, brown thrasher or a gray catbird. Control trials consisted of two Northern Cardinals placed in separate cages. These species have been documented as reservoirs

of WNv and are also five of the most abundant birds in the Atlanta area (20, 22). Birds were captured with 35 mm mesh polyester mist nets (Avinet, INC., Dryden, NY) in a study site near Emory University campus, Atlanta, Georgia. Captured birds were banded and weighted when banding equipment was available, aged and sexed before each experiment. Mist-netting aimed at collecting at least one northern cardinal and one of the experimental species listed above (experimental group) or two Northern Cardinals (controls). These experimental combinations represent the outcome of passive capturing of birds and we were unable to collect enough individuals to perform a full factorial design of host choice combinations. Northern cardinals were the most commonly captured species and thus chosen as the reference species due to the logistical constraints on capturing the numbers and types of wild bird species on any particular day. Birds used for experiments were housed on site in metal bird cages with water, a wooden perch, and either 100 grub worms or 50g birdseed depending on each species diet. Cages were placed in a dark and ventilated location until the experiment was conducted (the evening of the mist-netting day).

Thirty minutes after the official sunset time, the two metal cages containing the birds were placed on the floor of the aviary, spaced approximately 0.5 m apart (**Figure 1**). After 5 minutes of acclimation, 30 female mosquitoes were released into the enclosure; this marked the beginning of the experiment. Mosquitoes had free access to either bird, and the bird cages allowed the birds to defend themselves but not to escape the enclosure. Mosquitoes and birds were left in the aviary overnight, and the experiment ended at approximately 6:30 AM the following morning. Birds were first released from their cages and aviary using the stockinette entrance (cages remained in the aviary), and mosquitoes were then aspirated out of the enclosure with a Prokopack mosquito aspirator (Vazquez-Prokopec et al. 2009). An attempt was made to

quantify avian defensive behaviors with two Sony HandyCam video recorders; however short video lengths and poor visual quality prevented accurate estimations of avian defensive behaviors and defensive behaviors are not considered in the analyses.

Bloodmeal Analysis

All mosquitoes recovered from the aviary were euthanized at -4°C then scored as bloodfed or non-bloodfed (we observed no partial feeding) and individually preserved in a 1.5 ml cryovial with cell growth medium (Sigma-Aldrich, St. Louis, MO) at -80°C. Abdomens of blood fed mosquitoes were later dissected and blood DNA was purified using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer recommendations. Extracted DNA products were kept at -20°C. A fragment of the 16s ribosomal gene was amplified by direct PCR (Killmaster et al. 2011), products were run on a 1.5% ethidium bromide stained gel, and visualized under UV light. Successful PCR products were purified using a multiscreen plate purification kit (Millipore, Burlington, MA). DNA was sequenced in forward and reverse directions with an ABI 3500 (Applied Biosystems, Foster City, CA) automated sequencer. Sequences were assembled and reconciled using DNA Star Seqman Pro (DNASTAR, Madison, WI). A multi-sequence alignment with sample and control sequences was performed in BioEdit (Ibis Biosciences, Carlsbad, CA) and sequence comparisons were performed in MEGA5 (23).

The sequences obtained from mosquito blood meals were individually compared to reference sequences to establish the bird species upon which the mosquito had fed. Control reference sequences for each bird species used in this study were generated from blood clots from field collected birds targeting the same genetic marker. Sequence comparisons among bird species were performed in a 500 bp fragment of the 16S ribosomal gene using 76 variable sites that unmistakably discriminated all the bird species assayed in this work. Specifically, 38 sites

differentiated Northern Cardinals from American Robins, 44 sites differentiated from Blue Jays, 36 sites from Brown Thrashers, and 41 sites from Gray Catbirds. A > 98% base-pair match to any of the control sequences was used to determine which species the mosquitoes had fed.

The references sequences were compared with sequences available in Genbank that blasted with 100% homology to each of the target species except for Brown Thrasher, which we submitted to Genbank [Access numbers: Gray Catbird AF140866.1; Northern Cardinal AY 283884.1; Blue Jay KM042914; American Robin KJ909198.1; Brown Thrasher MH031275 – *this work*].

Data analyses

We analyzed two components of each experiments: overall feeding success and host choice. Because northern cardinals were used in all experiments, the reported outcomes of mosquito blood feeding success are attributed to the effect of the non-Cardinal hosts. The cardinal-cardinal experiments provided a baseline control to compare the overall blood feeding success of mosquitoes when presented with a non-Cardinal host species.

We utilized binomial generalized mixed-effects models (GLMMs) to investigate *Cx. quinquesfasciatus* blood feeding success and host preference. Analyses were performed at the level of each individual mosquito, reducing pseudoreplication. For blood feeding success (i.e., a mosquito blood fed or it did not), model selection compared feeding success among replicates using the non-Cardinal species as a fixed effect (with Cardinal-Cardinal experiments as the reference group), nighttime temperatures and relative humidity during a replicate as fixed effexts, and 'Replicate' as a random effect. Because we could not conduct a full factorial design of host species combinations, GLMMs comparing host choice among replicates (i.e., the mosquito fed on a Northern Cardinal or it did not) utilized only temperature and humidity as

fixed effects and replicate as a random effect. Mosquitoes from Cardinal-Cardinal replicates were not utilized in this analysis as the only host species available to mosquitoes was a Northern Cardinal. To aid model convergence, temperature and humidity were centered to their average value in the data set. All models were compared using AIC criteria. All GLMMs were implemented using the 'lme4' package for R, and all statistical analyses were performed in R (24).

Vector-host contact rates and vectorial capacity

The predicted host choice from the best fitting binomial GLMM was used to quantify vectorial capacity of *Cx. quinquefasciatus* mosquitoes for each experimental replicate. The formula for each bird species, *i*, is as follows:

$$C = - m_i * a_i^2 * h_i * P^{EIP} / \log(P)$$

where m = mosquito: host ratio, a = proportion mosquitoes blood fed on species i, P = mosquito nightly survival, EIP = WNV extrinsic incubation period, and h = probability species i transmits WNV infection to a mosquito. Certain values of the vectorial capacity equation were fixed: mosquito-host ratio was fixed at 14 mosquitoes per host to reflect the average number of mosquitoes released for each experiment; mosquito nightly survival was fixed at 0.9 which is similar to parameters used in previously published model of WNv transmission (25); and the extrinsic incubation period was fixed at 14 days which reflects previously published estimates (26). The probabilities of mosquitoes acquiring infections from hosts (h_i) were based on published estimates (27, 28). Because there are no published experimental studies of host competence for brown thrashers or gray catbirds, values published for the closely related northern mockingbird were used. All three species are in the same family, Mimidae.

Results

After a total of 110 mist netting hours, 34 birds were captured and utilized for 17 two-bird experiments: 3 northern cardinal-American robin, 2 northern cardinal-blue jay, 5 northern cardinal-brown thrasher, 3 northern cardinal-gray catbird, and 4 northern cardinal-northern cardinal controls (**Table 1**). A total of 460 *Cx. quinquefasciatus* mosquitoes were used for all experiments with an average of 27 mosquitoes released per replicate. A total of 387 (84.1%) mosquitoes were recaptured from the experiments; 219 (56.6%) were fully engorged (considered as sign of a successful blood meal). Blood meals from 154 mosquitoes were successfully sequenced and identified to a particular bird species. PCR products from 8 blood fed mosquitoes were unsuccessfully sequenced and 57 blood fed individuals from cardinal-cardinal control trials were not sequenced as northern cardinals were the only possible blood meal source. No mixed blood meals were detected by PCR.

Total blood feeding success ranged between 16.7 – 96% across all replicates (**Table 1).** Model comparisons using AIC criteria identified two candidate models for mosquito blood feeding success. The first model included humidity (centered to the median value in the data set) and experimental host combination as fixed effects with replicate as a random effect. Under this model, humidity improved the model's fit but there was no significant effect of humidity on total blood feeding success ($F = 1.13$, $p > 0.05$). With cardinal-cardinal combinations as the reference variable for model 1, there was no statistically significant difference in total mosquito blood feeding success between cardinal-robin, cardinal-jay, or cardinal-catbird combinations (**Table 1**); however, there was a significant difference in total mosquito blood feeding success was detected when the non-cardinal host was a brown thrasher (Odds ratio 0.22, 95% CI 0.05 – 0.98) (**Table 1**). The alternate model by AIC ($\Delta AIC < 1$) included only replicate as a random intercept term.

Under this model, blood feeding success varied considerably among replicates, and the odds of successful blood feeding were significantly different from one for numerous replicates (**Fig. 2**).

Model comparisons for mosquito host choice identified humidity and replicate was a random effect as the best model. Under this model, humidity had a small but significant predictor of blood feeding on a cardinal (F-value = 4.88, p = 0.03). The model additionally predicted no differences in the odds of blood feeding on a northern cardinal among replicates (**Fig. 3**).

Vectorial capacity estimates

Given published estimates for host competence for jays (C = 0.68), robins (C = 0.36), thrashers/catbirds (inferred from studies on northern mockingbirds, C = 0.15), and cardinals (C = 0.18), biting probabilities greater than 0.22 (for jays), 0.30 (for robins), 0.47 (for thrashers/catbirds), and 0.43 (for cardinals) generated capacity estimates greater than 1. Thus, despite not detecting a significant preference for any tested species, vectorial capacity estimates were largely driven by each host species' WNv competence index rather than host choice probabilities (**Fig. 4**). Comparing capacity estimates within each experimental combination, American robin estimates where significantly higher than cardinal estimates in 2 of 3 trials and blue jay capacity estimates in all trials were significantly higher than estimates for cardinals. Capacity estimates from cardinal-thrasher and cardinal-catbird were more variable and fewer estimates were significantly different from one another (**Fig. 4**). Despite such variable estimates of host choice, all host species combinations had the capability of generating total vectorial capacities estimates greater than 1.

Discussion

This study provides supporting, experimental evidence of *Cx. quinquefasciatus* opportunistic blood feeding behaviors. We did detect an effect of host species on overall blood

feeding success; specifically, brown thrashers were associated with an on overall lower feeding success rate in our experimental mosquitoes. Our experimental results detected no evidence for host preferences among the utilized host species; however, this could be an effect of low sample size and replication. Though we did not detect any significant host preferences for any of the species, vectorial capacity estimates were significantly different among host combinations, and this effect was largely driven by WNv host competence indexes. Our capacity estimates further suggest that northern cardinals, brown thrashers and gray catbirds can serve as sufficient amplifying hosts of WNv. Such findings support field evidence from the southeast that WNv is effectively amplified throughout avian populations with low to moderately competent host species (20).

Our study was designed to investigate variability in *Cx. quinquefasciatus* feeding behaviors when given a choice of two locally common host species. Our data cannot directly address how the presence of other host species may alter feeding patterns of *Cx. quinquefasciatus* individuals. *Culex quinquefasciatus* host feeding choices vary widely across North America, though in general the subspecies feeds opportunistically on avian hosts (29). In Central America, species in the order Galliformes (most likely chickens) are commonly identified blood meal hosts of *Cx. quinquefasciatus*; feeding on wild passerine species varies with 1.1% of hosts and approximately 20% of blood meal sources hosts identified to this order in Guatemala and the Yucatan, respectively (30, 31). In one study from Harris County, TX, U.S., over 60% of *Cx. quinquefasciatus* were identified as mammalian; in another, Northern Cardinals and Blue Jays accounted for over 45% of the identified avian blood meals (32, 33). Many avian species have been identified as blood meal hosts in California, and in one report House Finches, House Sparrows, and Mourning Doves accounted for approximately 72% of all avian identified

blood meals (34). In an experiment designed to quantify vector host preferences through the use of bird-baited field traps with passerine and Columbiformes species, wild *Culex quinquefasciatus* generally fed more frequently on the passerine species and showed a preference for house finches (35). In Memphis, TN, U.S., American robins were the most frequently identified blood meal hosts for *Culex quinquefasciatus* mosquitoes (19); American robins and northern cardinals were commonly identified blood meal hosts of *Culex* spp. mosquitoes from a study in Atlanta, GA, U.S. (20). Though our experiment does not capture the diversity of host species upon which *Cx. quinquefasciatus* feed, our results do provide evidence that specific combinations of bird species can generate variability in patterns of vector host choice as well as estimates of WNv transmission potential.

An important limitation of our study was our inability to separate the magnitude of *Cx. quinquefasciatus* host preferences from the avian defensive behaviors that can influence blood feeding success. However, we utilized low vector densities in order to increase the probability of successful blood meal acquisition and limit the impact of host defensive behaviors. Previous studies have shown defensive behaviors do not significantly limit mosquito blood feeding success. Darbro and Harrington (2007) determined that house sparrows were commonly fed upon by *Cx. pipiens pipiens* mosquitoes despite elevated defensive behaviors (36). A study by Edman et al. (1974) also did not observe mosquito host-switching from highly defensive hosts to less defensive hosts (37). Additionally, we cannot determine whether missing mosquitoes were eaten or not by hosts, though this is the most likely explanation for not recapturing all released mosquitoes. We also acknowledge the low statistical power of our experiments, which was due to the logistical difficulty of capturing specific wild host species in sufficient numbers to conduct all pair-wise combinations. While implementing analysis using GLMMs partially addressed this

limitation (38), we expect findings from our study can further the research of mosquito host choice in variable host species combinations and its relationship with pathogen transmission.

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Table 1. *Culex quinquefasciatus* blood feeding success for each experimental host combination.

Experimental Pair	Mosquitoes Released/ Retrieved/ Blood fed	% blood feeding (range)	Odds Ratio (95% CI) Overall blood-feeding success
Northern Cardinal & American Robin	71 / 61 / 42	68.9 (18.2 – 88.4)	0.85 (0.14, 4.83)
Northern Cardinal & Blue Jay	50 / 43 / 33	76.7 (50 – 96)	1.88 (0.25, 15.2)
Northern Cardinal & Brown Thrasher	141 / 122 / 43	35.2 (16.7 – 58.6)	0.22 (0.06, 0.98)
Northern Cardinal & Gray Catbird	78 / 70 / 44	62.9 (38.5 – 77.4)	0.90 (0.16, 5.13)
Northern Cardinal & Northern Cardinal	120 / 91 / 57	62.6 (40 – 92)	REFERENCE GROUP

Odds ratios were calculated using binomial GLMMs for 1) Overall mosquito blood feeding success, and 2) blood-feeding on Northern Cardinals. The reference group for each GLMM is listed within the table. Bolded cells indicate statistical significance at the $p < 0.05$ level.

*NA – not applicable

Figure Legends

Figure 1. Schematic of the constructed aviary for all experimental trials. The photos are of an American Robin (Cage 1) and Northern Cardinal (Cage 2) taken with a digital camera at the beginning of an experiment.

Figure 2. Odds ratio estimates for a binomial general linear mixed effects model of overall mosquito feeding success with only "replicate" as a random intercept term. Blue values indicate that the odds ratio point estimate is above 1 while the red values indicate the odds ratio point estimate is below one. Bolded values indicate odds ratio estimates are statistically different from 1. Abbreviations on the y-axis stand for the following combinations of species: NCAR – northern cardinal – American robin, NCBJ – northern cardinal – blue jay, NCBT – northern cardinal – brown thrasher, NCGC, northern cardinal – gray catbird, and NCNC – northern cardinal-northern cardinal. Numbers in these prefixed represent the replicate.

Figure 3. Odds ratio estimates for a binomial general linear mixed effects model of mosquito host choice with humidity as a random effect and "replicate" as a random intercept term. Blue values indicate that the odds ratio point estimate is above 1 while the red values indicate the odds ratio point estimate is below one. Abbreviations on the y-axis stand for the following combinations of species: NCAR – northern cardinal – American robin, NCBJ – northern cardinal – blue jay, NCBT – northern cardinal – brown thrasher, NCGC, northern cardinal – gray catbird, and NCNC – northern cardinal-northern cardinal. Numbers in these prefixed represent the replicate.

Figure 4. Vectorial capacity estimates using bootstrapped host choice probabilities from a binomial general linear mixed effects model with humidity as a fixed effect and replicate as a random effect. Density, Survival, extrinsic incubation period, and host competence indexes are listed in the text. The dashed horizontal line identifies capacity = 1. Abbreviations in the legend stand for the following combinations of species: NCAR – northern cardinal – American robin, NCBJ – northern cardinal – blue jay, NCBT – northern cardinal – brown thrasher, NCGC, northern cardinal – gray catbird, and NCNC – northern cardinal-northern cardinal. Open circles indicate estimates for cardinals while closed circles indicate estimates for the non-cardinal alternative. Bars represent 95% CI estimates of the vectorical capacity estimate.

Figure 1.

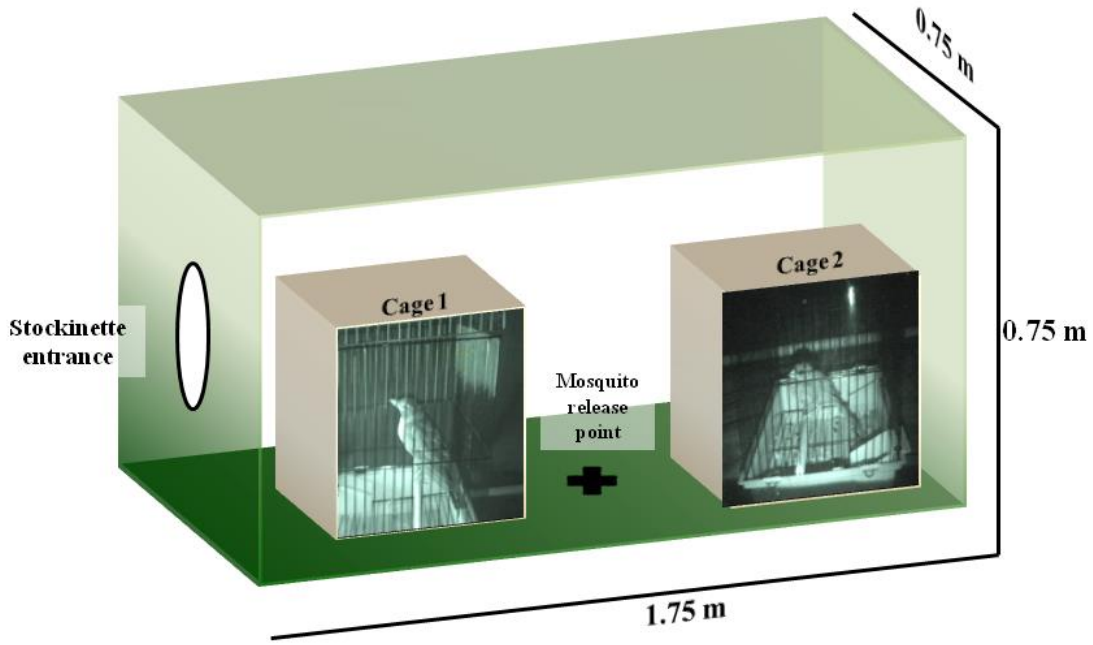

0.75 m
Stockinette entrance
Cage 1
Cage 2
Mosquito release point
0.75 m
1.75 m

Figure 2.

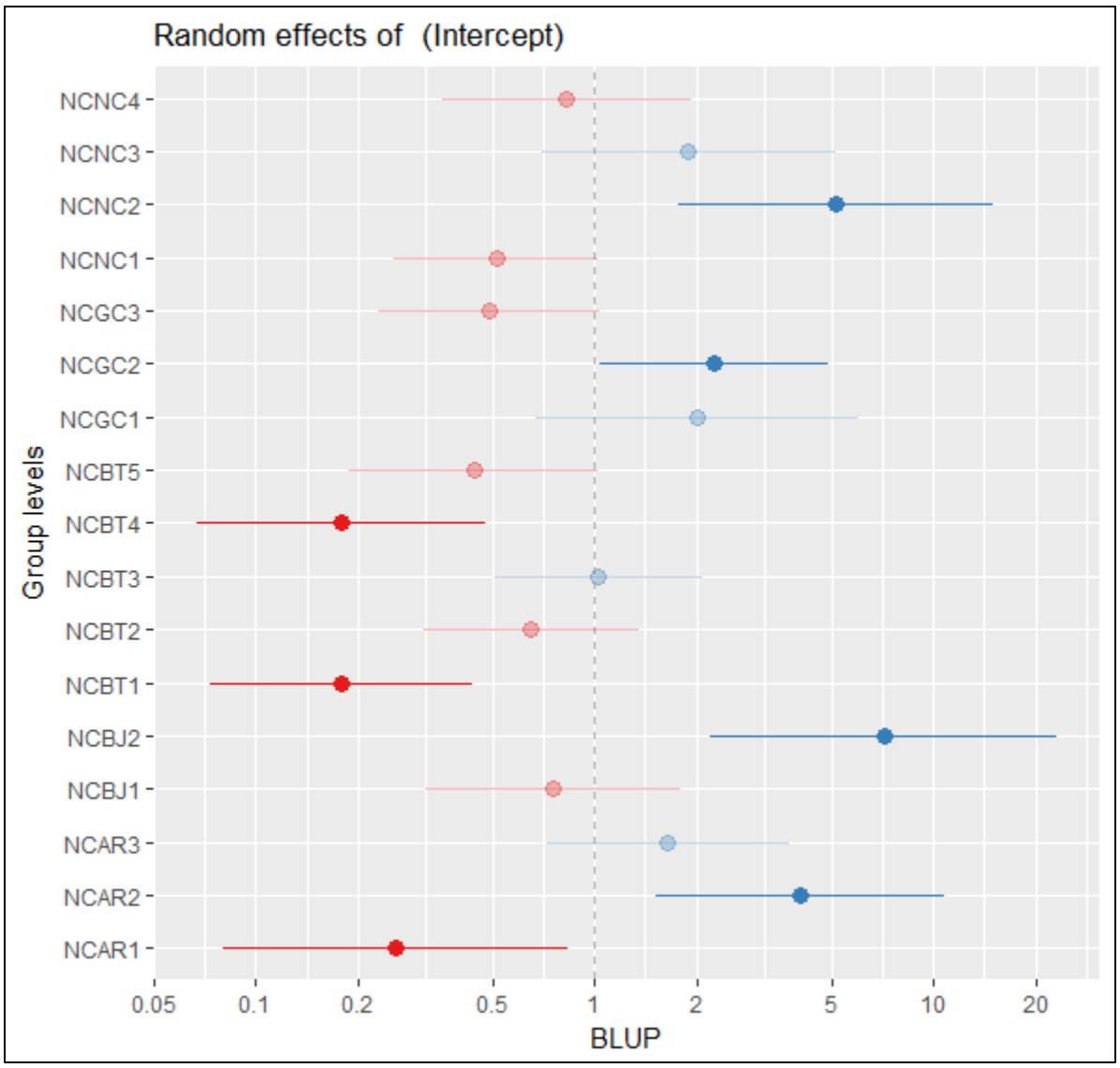

Random effects of (Intercept)
NCNC4
NCNC3
NCNC2
NCNC1
NCGC3
NCGC2
NCGC1
NCBT5
NCBT4
NCBT3
NCBT2
NCBT1
NCBJ2
NCBJ1
NCAR3
NCAR2
NCAR1
Group levels
0.05
0.1
0.2
0.5
1
2
5
10
20
BLUP

Figure 3.

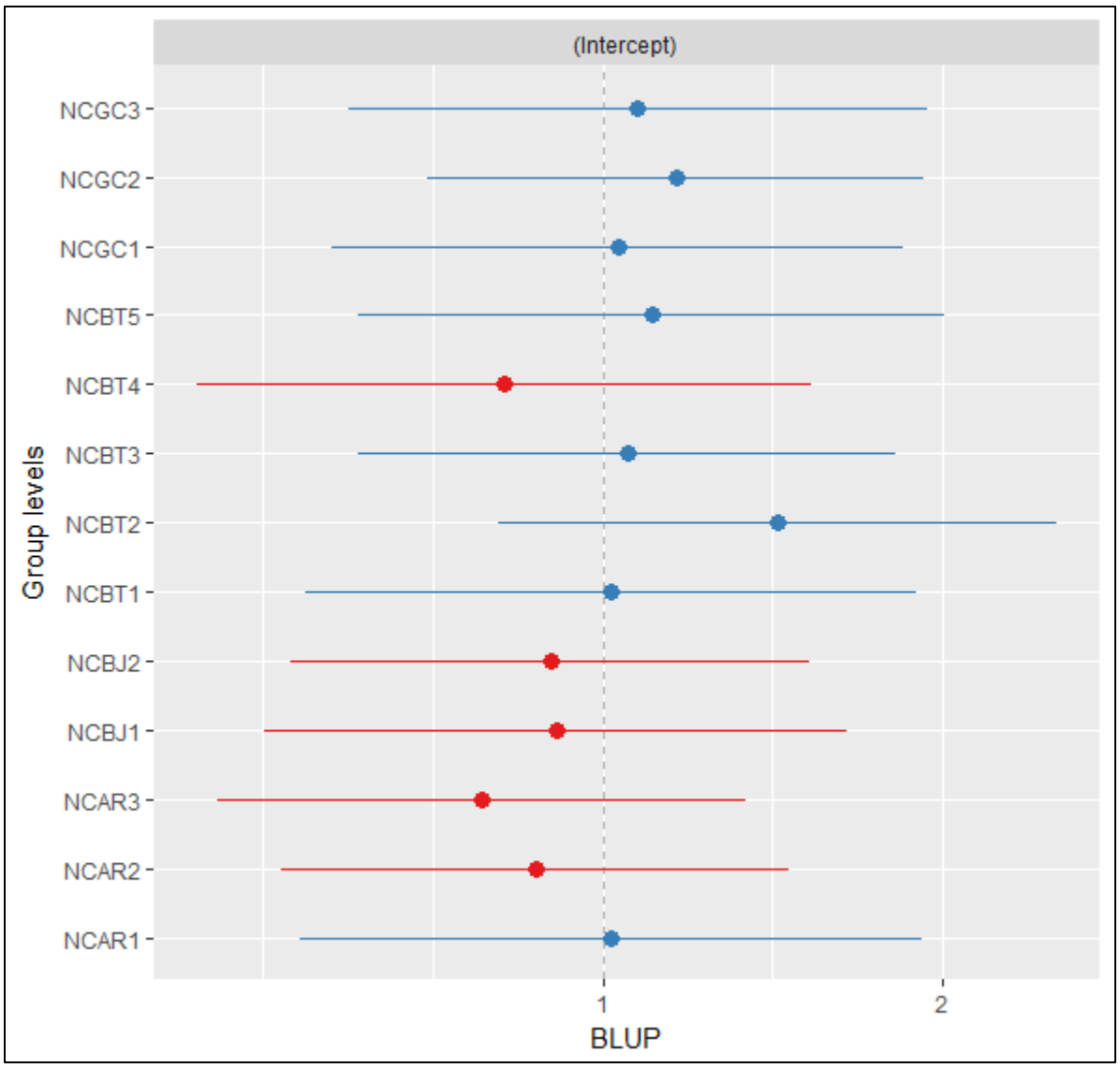
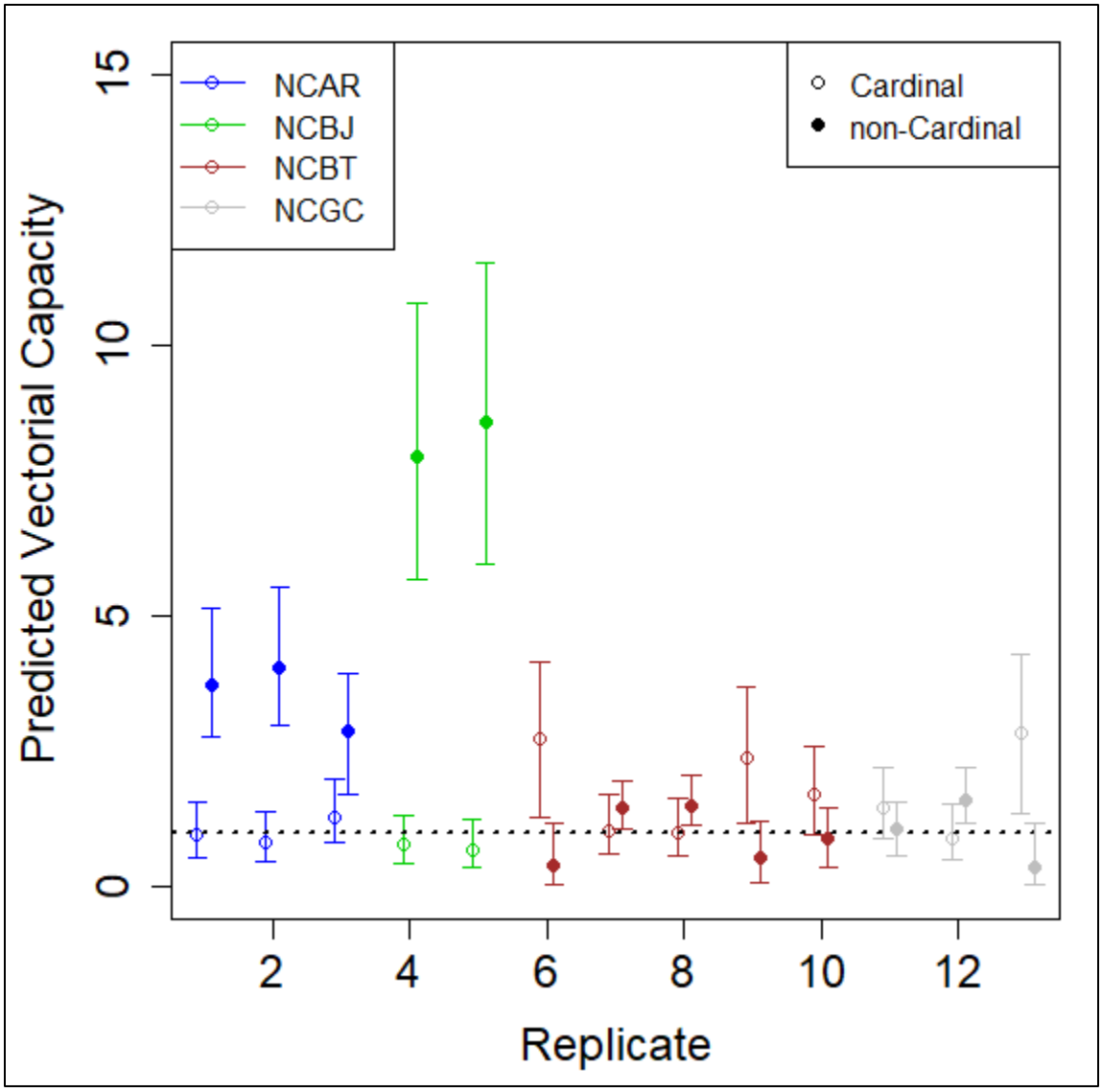

(Intercept)
NCGC3
NCGC2
NCGC1
NCBT5
NCBT4
NCBT3
NCBT2
NCBT1
NCBJ2
NCBJ1
NCAR3
NCAR2
NCAR1
Group levels
1
2
BLUP

Figure 4.


NCAR
NCBJ
NCBT
NCGC
Cardinal
non-Cardinal
Predicted Vectorial Capacity
0
5
10
15
2
4
6
8
10
12
Replicate

Chapter 4: Mosquito larval control and its impact on West Nile virus

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Introduction

West Nile virus (WNv) is a mosquito-borne zoonotic pathogen transmitted among birds by *Culex* spp. mosquitoes (1). The primary mosquito vectors include members of the invasive *Culex pipiens* complex, distributed as *Culex pipiens pipiens* in northern latitudes and *Culex quinquefasciatus* in southern latitudes. While humans and mammals are considered dead-end hosts of the virus, WNv exposure in humans can result in severe neurological symptoms and even death (1). Foci of WNv transmission occur predominately in urban and peri-urban environments (2, 3), and the occurrence of WNv epizootics in urban environments represents a threat to public health across the U.S.

The risk of WNv spillover to humans has been linked to ecological and landscape factors, including the presence and abundance of certain host and vector species as well as the presence of man-made waste water management systems, such as combined sewage overflows and road-side catch basins (4-8). Road-side catch basins are subterranean containers used to mitigate precipitation run-off from impermeable surfaces and are designed with a catchment that collects and prevents sediment and debris from entering underground water transportation systems. Such catchment reservoirs accumulate water that is often eutrophic and is an ideal breeding habitat for

Cx. pipiens complex mosquitoes (5, 9-12). Thus, catch basins represent an abundant and important target of vector control interventions, which often rely on the application of larvicides (13, 14).

Larvicides are an important tool for successful management of *Culex* spp. breeding population and are ideal for treating permanent and semi-permanent water sources (15). Because catch basins are abundant in cities and often contain breeding populations of *Cx. pipiens* complex mosquitoes, the application of larvicides in catch basins is a common form of mosquito control. The efficacy of larvicides in catch basins has been demonstrated previously (13, 14, 16); however, there is evidence that mosquito breeding populations within catch basins can persist despite repeated applications (17, 18). Additionally, there are no established epidemiological endpoints pertaining to the application of larvicides for mosquito control in the U.S. or Europe (19). Despite the widespread application of larvicides for WNv control, there is little empirical evidence that larviciding leads to effective reductions in adult *Culex* spp. populations and subsequent reductions in WNv-infected mosquitoes.

Here, we quantified the impact of applying two types of larvicides to suppress WNv transmission by *Culex* spp. mosquitoes, primarily *Cx. quinquefasciatus* and *Cx. restuans* in Atlanta, GA from 2015 to 2016. In 2015 the larvicide *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) was applied reactively (i.e., after the first detected WNv positive pool of any mosquito species) and in 2016 methoprene was applied proactively (i.e., at the beginning of the *Culex* spp. breeding season). Larvicide treatments were applied in previously identified foci of enzootic WNv transmission in Atlanta (5, 8) and coupled with WNv surveillance in mosquitoes in treated and untreated sites.

Methods

Study Locations

During 2015 and 2016, WNv mosquito surveillance took place weekly within four public parks in Atlanta, GA, USA: 1) Grant Park, GP, (sampled 04/01/15 – 12/14/16), 2) Phoenix Park, P3, (sampled 06/23/15 – 09/30/16), 3) Inman Park, IMP (sampled 03/21/16 – 12/14/16), and 4) Springvale Park, SVP, (sampled 3/21/16 – 09/30/16) (**Fig.1**). Each park is located in a census block previously identified as a hotspot of WNv transmission (5), and road-side catch basins are widely distributed within and along the road boundaries of each park. Residents near each sample site were informed of our experiment by contacting homeowners' associations.

Intervention Experimental Design

We adopted a Before-After-Control Intervention (BACI) design in which *Culex* spp. breeding populations and WNv transmission within a site were monitored before, during, and after an intervention. Observations within treatment sites were then compared within the site and between sites that received no treatment. Two different interventions were tested, a larviciding campaign reactive to the detection of WNv positive mosquito pools and a preventive campaign in which catch basins were prophylactically treated before the beginning of the WNv transmission season.

Reactive larval control

In 2015, breeding sites were surveyed within the road boundaries of GP; 36 permanent and semi-permanent possible breeding sites were discovered. *Bti* (Mosquito Dunks and Bits ©, Summit Chemicals, Baltimore, MD) products were applied weekly as 1 tablespoon of Mosquito Bits and 1 Mosquito Dunk in all breeding habitats from 07/17/15 to 09/02/15; larvicides were applied in basins whether they contained water or not to ensure full coverage within the

treatment area. The manufacturer's reported expected duration of efficacy of *Bti* applied as Mosquito Dunks © is 30 days; previous research with *Bti* products in catch basins note significant declines in efficacy after 1 week (14), so in this analysis we assumed duration of the insecticide to be only 1 week. Collections in P3 in 2015 were considered an un-manipulated control to collections in GP. Because GP and P3 were not sampled equally in 2015, the BACI period is considered to be from 07/17/15 to 11/03/15 which spans the 7 intervention weeks (including 1-week lag effect of last larvicide application) and 7 post-intervention weeks. Comparisons of collections within GP also consisted of a 7-week pre-treatment period spanning from 05/20/15 to 07/16/15.

Proactive larval control

In 2016, breeding sites in GP were resurveyed and treated biweekly with the juvenile growth hormone methoprene (Altosid©, Central Life Sciences, Schaumburg, IL), applied as 1 Altosid briquette per catch basin. We chose bi-weekly applications to minimize larvicide decay. Methoprene was also applied within SVP following the boundaries shown in **Fig. 1**. Because much of the space within SVP is private land, larvicide applications were restricted to road-side catch basins and breeding sites within public spaces. Methoprene was applied in basins from 03/20/2016 to 05/24/2016 whether they contained water or not to ensure full coverage within the treatment area; however, no larvicides were applied in GP or SVP the week of 5/16/16 due to heavy precipitation. The manufacturer's reported expected duration of efficacy of Altosid briquettes is 30 days; previous publications report sufficient coverage with methoprene up to 4 weeks (20, 21), so in this analysis we assumed duration of the insecticide to be 4 weeks. P3 and IMP received no larvicides during the 2016 surveillance season and are considered un-manipulated controls. The 2016 BACI period is considered to be from 03/20/16 to 09/30/16,

which spans 14 weeks of Altosid applications (including a 4-week lag-effect of last larvicide treatment) and 14 weeks of WNv mosquito surveillance.

Mosquito surveillance

Mosquito surveillance consisted of sampling *Culex* spp. breeding populations in road-side catch basins and collections of adult females with CDC gravid traps. Subsamples of 7 to 10 catch basins were chosen in each site for repeated weekly sampling after an initial survey of catch basins within each site. Each basin's interior was aspirated with a handheld Prokopack aspirator (22) for up to 5 minutes to collect resting adult mosquitoes. Additionally, three 300 mL water samples were collected with a modified dip cup and visually examined for pupae. If pupae were identified, samples were stored individually in 500 mL Whirl-Pack bags. Each basin's water depth was measured with a meter stick; in 2016 an YSI© Pro20 meter was used to record water temperature. CDC gravid trap collections took place weekly in all sites within 200 m of sampled catch basins. Between 3 and 5 gravid traps were baited with an infusion of dog food, hay, and tap water (23) and set in the evening after 5 PM and retrieved the following morning before 12 PM. All collections were returned to the Emory University laboratory where adult female mosquitoes were identified to species following a dichotomous key (24). Collected pupae were counted, placed in a BioQuip emergence chamber, and held at ambient lab temperature until emergence. After all pupae emerged, adults were euthanized at -4° C and identified to sex; females were identified to species following a dichotomous key (24).

WNv mosquito testing

All female mosquitoes identified to at least the genus level were pooled by date, collection method, collection site, and genus/species with up to 25 individuals per pool for WNv infection testing. Pools were tested for WNv using previously described virus isolation

techniques (8, 25). Minimum infection rates (MIR) per 1,000 individuals were estimated using the PooledInfRate plugin for Microsoft Excel© (26).

Data Analysis

Pupal and adult female *Culex* spp. collections in catch basins were chosen as the primary units for evaluating larvicide efficacy. Mixed effects models were used to analyze all data due to potential positive correlations between repeated spatial and temporal measurements. In all models 'Week' of collection and either 'catch basin' or 'gravid trap location' were modeled as random effects. For models analyzing the effect of larvicides within catch basins, 'Treatment' was modeled as a basin-specific categorical variable (1- Basin treated that week, 0 – Basin not treated that week). For models analyzing the effect of larval control on gravid trap collections and WNv minimum infection rates, 'Treatment' was modeled as a period-specific categorial variable: for 2015, 1 – Before treatment, 2 – During Treatment, 3 – After Treatment; for 2016, 1 – During Treatment, 2 – After Treatment.

For interventions applied reactively, we used Poisson-error generalized liner mixed models (GLMMs) to compare pupal and resting adult female collections in catch basins between treated (GP) and untreated (P3) sites while controlling for changes in basin water depth. We used negative-binomial error GLMMs to compare female *Culex* spp. gravid trap collections. To compare WNv mosquito minimum infection rates between treated and untreated sites, we first rounded MIR estimates to the nearest whole number and then implemented Poisson error GLMMs on the transformed variables.

For interventions applied proactively, we used binomial-error GLMMs to compare the proportion of pupae unable to emerge in catch basins with the number of collected pupae per basin as an offset variable while controlling for changes in basin depth and water temperature.

Poisson-error GLMMS were used to compare adult female collections from catch basins while controlling for changes in basin depth and water temperature. We then used negative-binomial error GLMMs and Poisson-error GLMMs to compare adult female collections in gravid traps and rounded MIR values similar to our analyses of reactively applied interventions.

All GLMMs were implemented using the 'glmer' function in the R package 'lme4' (27). All other analyses were performed in R V 3.4 (28).

Results

Reactive larval control

The application of *Bti* significantly reduced the number of collected pupae and adult female *Culex* spp. mosquitoes within treated catch basins during the reactive intervention period (**Table 1**). However, during the *Bti* application period (weeks 29 to 36) there were no significant differences in pupal collections between GP (*Bti* treated) and P3 (untreated) (Wilcoxon Test, W = 1366.5, p = 0.41) (**Fig. 2A**). Change in depth from the previous catch basin sample, a surrogate for precipitation, was not a significant factor affecting pupal or resting adult female *Culex* spp. mosquito collections (**Table 1**). There was a significant interaction between change in basin depth and *Bti* treatments possibly reflecting the combined effects of flushing due to precipitation and the killing power of the insecticide. Adult female *Culex* spp. collections in gravid traps were statistically higher in both sites before treatments were applied (**Table 2**). Collections after the treatment period were also significantly lower than before the treatment period (**Table 2**). Overall, there were no significant difference in CDC gravid trap collections between GP (treated) and P3 (untreated) during any experimental period (**Table 2, Fig. 2C**).

Proactive larval control

The application of methoprene significantly reduced the proportion of mosquitoes that successfully emerged as adults in treated (GP and SVP) versus untreated (P3 and IMP) sites (**Table 3**). We observed a long residual effect of methoprene (**Fig. 3A**), possibly due to frequent applications and a drought in the region during the study period. Accordingly, there was no significant difference in the proportion of pupae unable to emerge in treated basins during and after the methoprene application period (**Table 3**). Methoprene applications were associated with a small but significant decrease in adult female *Culex* spp. mosquito collections in treated catch basins (**Fig.3B**). However, site of collection had a far greater effect on adult *Culex* spp. collections within basins, and there were fewer resting *Culex* spp. mosquitoes in IMP, P3, and SVP catch basins compared to GP (**Table 3**). This significant effect of site was in spite of GP being treated with methoprene. There was a significant difference in adult female *Culex* spp. collections in gravid traps between IMP and GP in the AFTER-treatment period of the study; there were no significant differences in any site in the DURING-treatment period (**Table 4, Fig. 3C**).

WNv mosquito infections

Evidence of WNv transmission was detected in weeks 27 – 35 in 2015 ('During' the *Bti* application period, **Fig. 2D**) and in weeks 27 – 39 in 2016 ('After' the methoprene application period, **Fig. 3D**). In 2015, peak mosquito infection occurred during weeks 28 and 30 for P3 (untreated) and GP (treated), respectively (**Fig. 2D**). In 2016, peak mosquito infection occurred during weeks 33, 34, 35, and 35 for SVP (treated), IMP (untreated), GP (treated), and P3 (untreated), respectively (**Fig. 3D**). Because the detection of WNv was similar between all sites, we did not include 'Treatment Period' as a fixed effect in any GLMM. Regardless of whether larvicides were applied reactively or proactively, the duration, onset, and timing of peak WNv

infection in adult *Culex* spp. mosquitoes was similar between treated and untreated sites (**Table 2 and 4, Fig. 2D and 3D**).

Discussion

Our results show that within catch basins, larvicides such as *Bti* and methoprene are effective at reducing breeding populations of *Culex* spp. mosquitoes within this particular breeding environment. However, we were unable to link effective mosquito larval control to significant reductions in adult female mosquito populations either resting within basins just above the water line or in gravid traps in close spatial proximity to treated catch basins. Importantly, we did not detect any difference in rates of WNv mosquito infections in treated versus untreated sites during either the reactive or proactive intervention periods, thereby indicating that these methods, following our application procedures and spatial coverage, were insufficient to suppress epizootic WNv transmission in our study areas.

Previous attempts to link larval control to reductions in adult *Culex* spp. mosquito populations have yielded mixed results. In Chicago, IL, U.S., counts of adult mosquitoes in gravid traps were positively correlated with larval collections in catch basins 1 week prior (13), yet there was no evidence that fluctuations in adult mosquito collections were due to larviciding treatments. Larval source management, i.e., draining or total eliminating mosquito breeding habitats, is equally difficult to demonstrate as an effective control tool for *Culex* mosquito-borne diseases. However, a recent report on large-scale removal of invasive honeysuckle did link the removal of this plant to reductions in *Culex* spp. populations within removal sites (29). In our study, *Culex* spp. pupal declines and mortality were significantly associated with *Bti* and methoprene applications, respectively. However, we detected only marginal reductions in the number of *Culex* spp. adult females resting in catch basins and no significant declines in gravid

trap collections. We also found no difference in WNv infection rates in mosquitoes in treated sites compared to untreated sites. Because there were numerous breeding sites untreated outside of and possibly within the boundaries of our treatment sites, much greater coverage of insecticides across time and space may be needed to show an effect of larval control on mosquito-borne zoonotic pathogens such as WNv.

In general, the U.S. Centers for Disease Control and Prevention (CDC) does not recommend using larvicides or larval source reduction techniques as the sole means to reduce rates of epidemic WNv transmission (15). Currently, aerially applied adulticides are the only tools purportedly shown to have reduced the prevalence of WNv infections in *Culex* spp. mosquitoes as well as the risk of WNv exposure in humans (30, 31). A 2012 analysis of aerial applications of insecticides to control human exposure to WNv during an outbreak in Dallas-Fort Worth, TX, U.S., revealed that declines in the incidence of human WNv exposure occurred in both treated versus untreated sites (32). Aerial insecticide applications are only recommended as an emergency response, and public health agencies are encouraged to practice larval control year-round in hopes that these efforts will mitigate the need for emergency measures (15). Importantly, there are no accepted epidemiological endpoints for the use larvicides to control mosquito-borne pathogens (19) – meaning it is assumed that larvicides have some effect on pathogen transmissio by mosquitoes and there is little empirical evidence of expected impact given a specified insecticide treatment. Mosquito control guidelines across the U.S. would benefit from more thorough studies with defined epidemiologic end-points in order to best inform the use of larvicides for control mosquito-borne pathogens.

A limitation of our study is that we do not know the spatio-temporal scale at which WNv enzootic transmission occurs in the region. The similarity of weekly WNv mosquito infection

estimates between treated and un-treated sites across years and sites suggests that WNv transmission in the region may be occurring on a much larger scale than what was treated (average treatment area < 1 km^2). Previous analyses of WNv in Chicago, IL, U.S. show that circulating viral populations in mosquitoes can represent an admixture of locally-derived and introduced viruses (33). Similarly, in Chatham County, GA, U.S., detection of local WNv circulation went below detectable levels in the enzootic cycle from 2008 – 2010 only to be re-introduced in 2011 with a variant similar to one circulating in the northeastern U.S. in 2008 - 2009 (34). The reported dynamics of the virus indicate that localized patterns of WNv transmission represent not only focal transmission events between mosquitoes and birds but also large-scale movements of the virus most likely by infectious hosts. These fine and coarse-scale viral dynamics should be considered when conducting WNv surveillance and control at local scales.

The production of mosquitoes from cryptic breeding habitats and/or private property within and near treatment sites may have also limited our ability to effectively suppress *Culex* spp. populations. Because *Cx. pipiens* complex mosquitoes are capable of traveling up to 1.5 km a night (35), it is likely that mosquitoes produced outside treatment sites were captured within treatment sites. Coupling larviciding with community outreach that informs and encourages residents to reduce breeding habitats within property boundaries may increase the coverage of mosquito larval control and increase population reductions (36).

Road-side catch basins and storm drains are ubiquitous in urban and peri-urban environments, and *Culex* spp. mosquitoes are known to proliferate within these man-made structures. We suggest that coordinated efforts between local vector control agencies that synchronize and extend the spatio-temporal scale of larvicide applications may better impact

local cycles of WNv enzootic transmission. We also advocate for the extension of entomologic evaluations of mosquito larval control above the water line and the adoption of epidemiological endpoints that clearly define expected levels of vector population reductions and pathogen control.

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Table 1: Results from a Poisson-error general linear mixed effects model for the abundance of pupae and adult female *Culex* spp. mosquitoes in sampled catch basins during the *reactive* larval control experiment. Grant Park is the reference site.

	Collected Pupae				**Collected Adult Female *Culex* spp.**			
Variable	*Est.*	*S.E.*	*Z value*	*Pr(> |z|)*	*Est.*	*S.E.*	*Z value*	*Pr(> |z|)*
Intercept	0.15	0.77	0.20	0.84	1.41	0.46	3.07	0.002
Treatment: Bti	-2.99	1.13	-2.65	0.008	-0.43	0.44	-0.98	0.33
Site**:** P3	-2.23	1.13	-1.97	0.05	-2.66	0.66	-4.01	< 0.001
Change in basin depth	-0.02	0.007	-2.35	0.02	0.005	0.006	0.87	0.38
Interaction (Depth change & Treatment)	0.06	0.04	1.61	0.11	-0.05	0.02	-3.10	0.002
Random Effects (obs. 235)	*Var.*	*S.D.*			*Var.*	*S.D.*		
Catch Basin (n = 17)	1.96	1.40			1.05	1.03		
Week by Park (n = 35)	4.28	2.07			0.86	0.93		

Table 2: Results from a Negative-binomial general linear mixed effects model for adult *Culex* spp. collections in gravid traps and a Poisson GLMM for Trap-specific WNv Minimum Infection Rate during the 2015 experimental period. Grant Park is the reference site while Treatment Period: Before is the reference period.

	Variable	*Estimate*	*S.E.*	*Z value*	*Pr(> |z|)*
Adult Female Collections	Intercept	4.79	0.21	22.5	< 0.001
	Treatment Period: During	-1.21	0.30	-4.05	< 0.001
	Treatment Period: After	-1.52	0.30	-5.09	< 0.001
	Site: P3	0.26	0.43	0.60	0.55
	Interaction: P3 * Period: During	-0.44	0.54	-0.82	0.41
	Interaction: P3 * Period: After	0.31	0.54	0.59	0.56
	Random Effects (obs. 131)	*Var.*	*S.D.*		
	Trap Site (n = 8)	0.02	0.13		
	Week by Park (n= 22)	0.21	0.46		

	Variable	*Estimate*	*S.E.*	*Z value*	*Pr(> |z|)*
WNv Minimum Infection Rate	Intercept	-4.58	2.30	-1.99	0.05
	Site: P3	-2.01	2.25	-0.90	0.37
	Random Effects (obs. 131)	*Var.*	*S.D.*		
	Trap Site (n=8)	2.14	1.46		
	Week by Park (n= 39)	17.2	4.15		

Table 3: Results from binomial general linear mixed effects model (GLMM) for the proportion of pupae unable to emerge as adults and Poisson GLMM results for the number of collected adult female *Culex* spp. mosquitoes in sampled catch basins during the 2016 experimental period. Grant Park is the reference site. Water temperature is centered to the mean value in the data set.

	Un-emerged Pupae				**Collected Adult Female *Culex* spp.**			
Variable	*Est.*	*S.E.*	*Z value*	*Pr(> |z|)*	*Est.*	*S.E.*	*Z value*	*Pr(> |z|)*
Intercept	1.62	0.67	2.42	0.02	1.69	0.32	5.25	< 0.001
Treatment: Altosid	0.59	0.71	0.83	0.41	0.12	0.27	0.43	0.67
Site: Inman Park	-4.17	0.98	-4.27	< 0.001	-0.96	0.46	-2.07	0.04
Site: Phoenix Park	-4.41	1.03	-4.29	< 0.001	-1.52	0.47	-3.23	0.001
Site: Springvale Park	-0.20	0.95	-0.22	0.83	-1.52	0.44	-3.48	< 0.001
Water Temp**.** (centered)	-0.10	0.07	-1.47	0.14	0.14	0.02	5.85	< 0.001
Change in Basin Depth	-0.01	0.01	-2.00	0.05	-0.01	0.003	-3.62	< 0.001
Interaction: Treatment * Depth	0.06	0.03	1.88	0.06	0.01	0.006	2.51	0.01
Random Effects (Pupae: obs. 339) (Adults: obs. 706)	*Var.*	*S.D.*			*Var.*	*S.D.*		
Catch Basin (n = 33)	2.24	1.50			0.64	0.80		
Week by Park (Pupae: n= 86) (Adults: n = 98)	2.99	1.73			0.59	0.77		

Table 4. Results from a negative-binomial general linear mixed effects model for adult female *Culex* spp. gravid trap collections and a Poisson GLMM for Trap-specific WNV minimum infection rate during the 2016 experimental period. Grant Park is the reference site and Treatment Period: During is the reference experimental period.

	Variable	*Estimate*	*S.E.*	*Z value*	*Pr(> |z|)*
Adult Female Collections	Intercept	4.39	0.19	23.28	< 0.0001
	Treatment Period: *After*	0.07	0.26	0.28	0.78
	Site: Inman Park	-0.53	0.28	-1.87	0.06
	Site: Phoenix Park	-0.43	0.29	-1.49	0.14
	Site: Springvale Park	-0.30	0.30	-1.01	0.31
	Interaction: IMP * Period: After	1.01	0.39	2.59	0.01
	Interaction: P3 * Period: After	0.53	0.40	1.32	0.19
	Interaction: SVP * Period: After	0.67	0.41	1.64	0.10
	Random Effects (obs. 421)	*Var.*	*S.D.*		
	Trap Site (n = 14)	0.0	0.0		
	Week (n= 110)	0.37	0.61		
	Variable	*Estimate*	*S.E.*	*Z value*	*Pr(> |z|)*
WNv Minimum Infection Rate	Intercept	-6.55	1.43	-4.58	< 0.0001
	Treatment Period	*NA*	*NA*	*NA*	*NA*
	Site: Inman Park	1.74	1.70	1.03	0.30
	Site: Phoenix Park	-0.14	1.46	-0.10	0.92
	Site: Springvale Park	0.05	1.45	0.03	0.97
	Random Effects (obs. 421)	*Var.*	*S.D.*		
	Trap Site (n=14)	0.20	0.44		
	Week by Park (n= 110)	46.8	6.84		

Figure Legends

Figure 1. West Nile virus surveillance site map.

Figure 2. Reactive larval control and WNv surveillance by site in 2015. A) Number of collected pupae in catch basins, B) Number of resting adult female *Culex* spp. mosquitoes in catch basins, C) Number of *Culex* spp. female mosquitoes in gravid traps, and D) Weekly WNv minimum infection rates (per 1,000 individuals). The vertical dashed lines in each plot delineate the 'Before', 'During', and 'After' *Bti* treatment periods.

Figure 3. Proactive larval control and WNv surveillance in 2016. Average collections from Treatment (GP/SVP) and Control (IMP/P3) sites are shown in all plots to improve data visualization. A) Average proportion of pupae unable to emerge as adults, B) Average number of resting adult female *Culex* spp. mosquitoes, C) Average number of *Culex* spp. female mosquitoes in gravid traps, and D) Weekly WNv minimum infection rates (per 1,000 individuals). Vertical dashed line in each plot delineate the 'During' and 'After' methoprene treatment periods.

Figure 1.

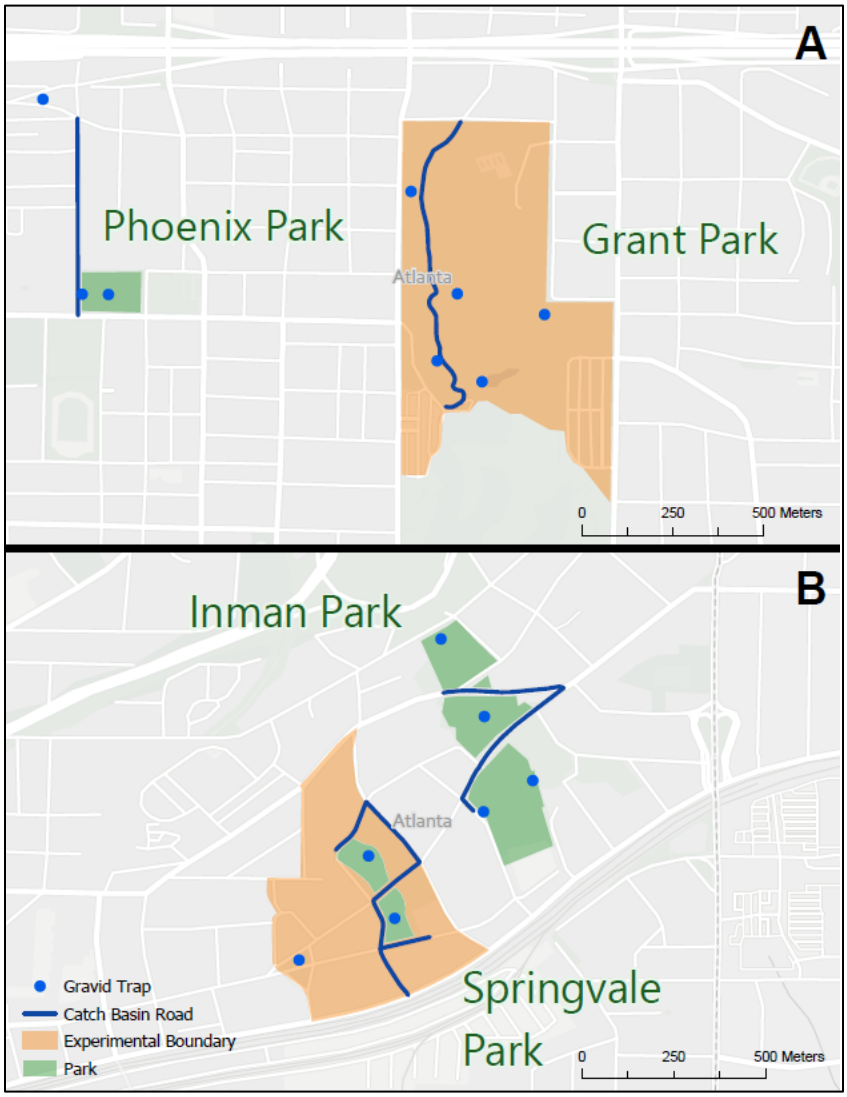

A
Phoenix Park
Grant Park
Atlanta
0
250
500 Meters
B
Inman Park
Atlanta
Springvale
Park
Gravid Trap
Catch Basin Road
Experimental Boundary
Park
0
250
500 Meters

Figure 2.

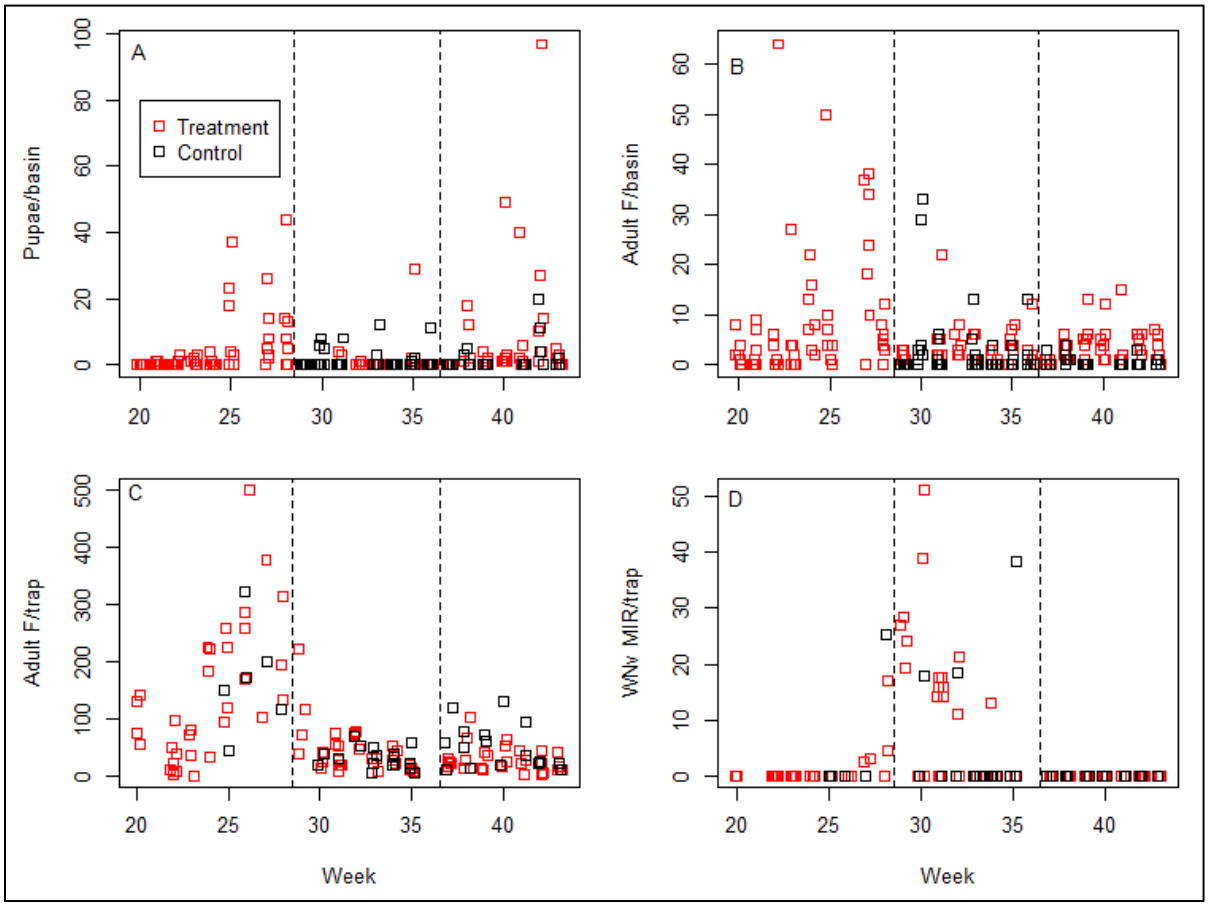
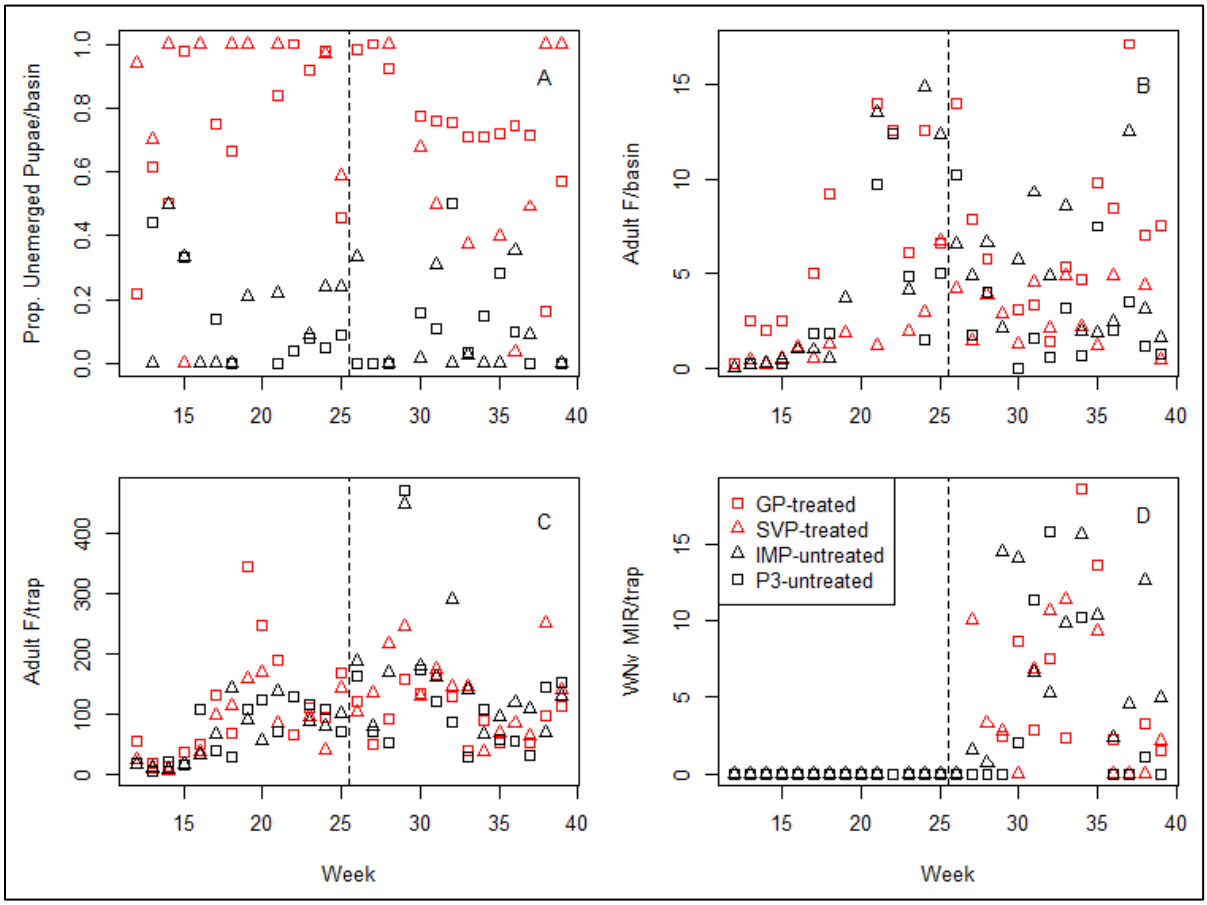

A
Treatment
Control
Pupae/basin
B
Adult F/basin
C
Adult F/trap
D
WNv MIR/trap
Week
Week

Figure 3.


Prop. Unemerged Pupae/basin
A
Adult F/basin
B
Adult F/trap
C
WNv MIR/trap
D
GP-treated
SVP-treated
IMP-untreated
P3-untreated
Week
Week

Conclusions

Summary

The objective of my dissertation was to quantify sources of WNv transmission heterogeneity attributable to the composition and structure of vector and host species communities. In Chapter 1, I used comprehensive field surveillance of WNv transmission in Atlanta, GA, U.S. coupled with a temperature-dependent vectorial capacity model to quantify the likelihood that two vector species, *Cx. restuans* and *Cx. quinquefasciatus*, contribute to local patterns of WNv transmission. I found that the majority of empirical evidence regarding WNv transmission incriminated *Cx. quinquefasciatus* as the primary vector of WNv transmission. My temperature-dependent vectorial capacity model also suggested that *Cx. restuans* is unlikely to be an efficient vector of WNv because temperatures during this species' period of greatest field abundance are unfavorable for within-vector viral replication. The availability of susceptible hosts was also very low during *Cx. restuans'* temporal distribution in the field, further limiting this species' likelihood of participating in WNv transmission. Additionally, abundant *Cx. restuans* populations were limited to a single site in the city (GP), suggesting that in general this species may not be an abundant and important vector of WNv in urban environments in the southeastern U.S.

In Chapter 2, I quantified species-specific contributions to the prevalence of WNv infections in host communities in both Atlanta, GA and Chicago, IL, U.S. through the use of long-term avian serosurveys. Using an empirical approach, I identified variability in the annual cumulative force of infection (FOI) for WNv between numerous host species. I defined the cumulative force of infection as the accumulated probability a susceptible individual (in my

study hatch year birds) encounters WNv during a transmission season. In general, FOI estimates were lower across all sampled years and species in Chicago, IL compared to Atlanta, GA. Within each city, northern cardinals had the highest predicted probability of encountering WNv, possibly suggesting that this species is a much more important host of WNv than predicted from *Cx. pipiens pipiens* and *Cx. quinquefasciatus* blood meal studies (1, 2).

In Chapter 3, I used controlled host choice experiments to estimate *Cx. quineufasciatus* feeding preferences for the following hosts of WNv in Atlanta, GA (3): American robins, northern cardinals, brown thrashers, gray catbirds, and blue jays. The overall feeding success of *Cx. quinquefasciatus* mosquitoes was significantly influenced by the experimental combination of hosts, and mosquitoes were less likely to blood feed when northern cardinals were paired with a brown thrasher. However, due to the limitations of our experimental design, we were unable to detect a preference for any species by blood feeding *Cx. quinquefasciatus* mosquitoes. Vectorial capacity models that included my experimental blood feeding results predicted that host competence was the primary driver of species specific contributions to WNv transmission. However, capacity estimates across all replicates predicted that WNv transmission is likely in all experimental host species combinations.

Finally, in Chapter 4 I conducted an applied study and tested the ability of larvicides to suppress enzootic WNv transmission. This study was conducted across two field seasons: one during which the larvicide, *Bti*, was applied re-actively (after the detection of WNv infections in mosquitoes) and one during which the larvicide, methoprene, was applied proactively (at the beginning of the mosquito breeding season). Larvicides proved to be an effective tool to control breeding populations of *Culex* spp. mosquitoes (i.e., larvae and puape). However, I detected no

differences in either the number of female mosquitoes collected in CDC gravid traps or the prevalence of WNv infections in mosquitoes between treated and untreated sites.

West Nile virus is a complex wildlife disease that is transmitted among numerous vector and host species that differ in their ability to acquire and transmit the virus as well as their distributions across the landscape (4-6). Theory indicates that the local composition of host and vector communities is an important determinant of transmission (7), and by using both field and experimental approaches, I have demonstrated numerous species-specific contributions to WNv transmission in Atlanta, GA. This research both confirms previous reports on WNv transmission in the southeastern U.S. as well as generates new insights on species-specific contributions to WNv transmission in the southeast. However, by field evidence indicates that the intensity of WNv epidemics and enzootics can vary between regions in the U.S. with similar compositions of species (see Chapter 2). Additionally, the intensity of WNv epidemics can also appear similar between regions with different compositions of species (8, 9). Further ecological and epidemiological research may be needed to identify the within-host and vector processes of infection that drive such similarities and dissimilarities in patterns of WNv transmission.

Future directions

Pathogen surveillance techniques such as those employed in Chapters 1 and 2, are common tools to investigate the dynamics of transmission at local scales, yet surveillance techniques have many limitations. For one, surveillance is subject to sampling bias (10). Additionally, surveillance methods for pathogens such as WNv are often designed to capture specific types of individuals or are employed to investigate a risk factor of human incidence

rather than to investigate the ecological dynamics of wildlife transmission. I propose that experimental approaches such as those described in Chapters 3 and 4 may prove a more powerful tool for investigating certain species-specific contributions to WNv transmission.

Following on the experimental design in Chapter 3, controlled studies could be expanded to empirically test the effects of host abundance on vector feeding behaviors. Though I was unable to detect a feeding preference for any host species by *Cx. quinquefasciatus,* a stronger test of preference would be to pair a single individual of one species with numerous individuals of another species. These types of studies could also manipulate the diversity of vector species to estimate the extent of blood meal hosts overlap between primary and secondary vector species. Though Chapter 4 attempted to utilize insecticides to suppress mosquito populations, any exclusion technique could be applied in natural or semi-natural settings to manipulate the presence and abundance of certain vector and host species in the environment. Similar exclusion techniques have been used to study the effect of large mammal exclusions on rodent-borne diseases (11) as well as colonization and extinction rates of arthropods on islands (12-14). For WNv, manipulative cage experiments have been used to quantify per capita biting rates in different host group formations (15). Following the methodological approach in Chapter 4, insecticides and/or devises such as predator mimics could be used in conjunction with pathogen surveillance in treated and untreated sites to quantify the effect of the suppression or exclusion approach on metrics of WNv transmission. Exclusion studies of these sorts, though difficult to conduct, may provide valuable insights into topics such as the indirect effects of predator presence on pathogen transmission. In more controlled settings, experiments could be used to test whether the addition of multiple vector species leads to an additive (linear) or multiplicative (non-linear) change in host infection prevalence.

Additionally, during the course of my research it has come to my attention that there are no published epidemiological endpoints for larval control interventions for *Culex* spp. transmitted diseases such as WNv (8) – this is despite the general recommendation from the CDC that local mosquito control agencies (and the public in general) practice *Culex* spp. larval control year round (16). Larvicides work, at least from the standpoint that they effectively kill mosquito larvae and pupae. Also, larvicides or larval control accompanied with other mosquito control techniques can have powerful effects on disease suppression (17). However, it is unknown what the spatial and temporal coverage of larvicide treatments needs to be in order to effectively suppress blood feeding *Culex* spp. populations to levels insufficient to transmit WNv. In addition to this knowledge gap concerning the efficacy of larval control, there are few studies that can effectively quantify an impact of adult *Culex* spp. mosquito control on the prevalence of WNv infections in either mosquitoes or humans (18-20). The public health field would greatly benefit from large-scale, controlled field experiments with specified insecticide coverages and clearly defined metrics of disease transmission and incidence. Investigations of the sort would be expensive and time consuming, and there are many ethical considerations to take into account regarding where and when larvicides are applied. However, the results from such experiments would be scientifically, epidemiologically, and monetarily invaluable.

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